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SOFT TISSUE CALCIUM LEVELS DURING AGING IN HIGHLY INBRED RATS

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DISSERTATION COMMITTEE

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TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF ILLUSTRATIONS	viii
Chapter	
I. INTRODUCTION	1
Aim of This Study	1
Processes Associated with Aging	3
Calcium	10
Atomic Absorption Spectrophotometry	15
II. MATERIALS AND METHODS	18
Animals	18
Methods	22
III. RESULTS	37
Findings Within the Strains	37
Findings Between Strains	69
Summary of Results	87
IV. DISCUSSION	89
Aging Changes in Dynamic Equilibrium of Calcium	90
Correlation of Dynamic Equilibrium with Changes in Tissue Calcium Contents	93
Relationship of Tissue Calcium Changes to Chronological and Physiological Age	96
Application of the Tissue Calcium Changes to Various Functions	98
V. SUMMARY AND CONCLUSIONS	101
BIBLIOGRAPHY	105
APPENDIXES	114

LIST OF TABLES

Table		Page
1.	Effect of Perfusion with Isotonic Sucrose on Tissue Calcium Content	24
2.	Instrument Settings for Determination of Calcium	31
3.	Calcium Recovery from a Sample of Known Calcium Concentration	34
4.	New Multiple Range Test for Kidney Calcium Content in Fischer and A x C Rats	42
5.	New Multiple Range Test for Lung Calcium Content in Fischer and A x C Rats	44
6.	New Multiple Range Test for Cerebral Cortex Calcium Content in Fischer and A x C Rats	48
7.	New Multiple Range Test for Skin Calcium Content in Fischer and A x C Rats	51
8.	New Multiple Range Test for Aorta Calcium Content in Fischer and A x C Rats	53
9.	New Multiple Range Test for Cardiac Muscle Calcium Content in Fischer and A x C Rats	55
10.	New Multiple Range Test for Skeletal Muscle Calcium Content in Fischer and A x C Rats	57
11.	New Multiple Range Test for Liver Calcium Content in Fischer and A x C Rats	61
12.	New Multiple Range Test for Intestinal Smooth Muscle Calcium Content in Fischer and A x C Rats	63
13.	New Multiple Range Test for Plasma Calcium Content in Fischer and A x C Rats	65
14.	New Multiple Range Test for Whole Blood Calcium Content in Fischer and A x C Rats	67
15.	Total Percentage of Difference in Tissue Calcium Content and Chronological Age When the Change is Achieved in Fischer and A x C Rats	70
16.	Comparison of Kidney Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	72

LIST OF TABLES -Continued

Table		Page
17.	Comparison of Lung Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	74
18.	Comparison of Cerebral Cortex Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	75
19.	Comparison of Skin Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	76
20.	Comparison of Aorta Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	77
21.	Comparison of Cardiac Muscle Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	78
22.	Comparison of Skeletal Muscle Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	79
23.	Comparison of Liver Calcium Contents in Fischer and A x C Rats at Corresponding periods of the Respective Life Spans	81
24.	Comparison of Intestinal Smooth Muscle Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	82
25.	Comparison of Plasma Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	83
26.	Comparison of Whole Blood Calcium Contents in Fischer and A x C Rats at Corresponding Periods of the Respective Life Spans	84
27.	Summary of Comparisons between the Tissue Calcium Contents of the Two Rat Strains at Equivalent Physiological Ages	85
28.	Comparisons of Overall Mean Tissue Calcium Contents in Fischer and A x C Rats	87

LIST OF ILLUSTRATIONS

Figure		Page
1.	Mean Body Weight in Grams of Fischer and A x C Rats During the Life Span	20
2.	Emission Spectrum of the Calcium Hollow Cathode Lamp	29
3.	Kidney Calcium Concentration as a Function of Age	41
4.	Lung Calcium Concentration as a Function of Age	43
5.	Cerebral Cortex Calcium Concentration as a Function of Age	47
6.	Skin Calcium Concentration as a Function of Age	50
7.	Aorta Calcium Concentration as a Function of Age	52
8.	Cardiac Muscle Calcium Concentration as a Function of Age	54
9.	Skeletal Muscle Calcium Concentration as a Function of Age	56
10.	Liver Calcium Concentration as a Function of Age	60
11.	Intestinal Smooth Muscle Calcium Concentration as a Function of Age	62
12.	Plasma Calcium Concentration as a Function of Age	64
13.	Whole Blood Calcium Concentration as a Function of Age	66

SOFT TISSUE CALCIUM LEVELS DURING AGING IN HIGHLY INBRED RATS

CHAPTER I

INTRODUCTION

The electrolyte pattern in mammals is known to undergo marked changes during aging, but interpretation of the changes is difficult to accomplish because of the contradictory nature of the results which have been reported (Korenchevsky, 1961). One of the electrolyte patterns that has been of interest to investigators of aging processes is that exhibited by calcium. There is not uniform agreement, however, even as to the qualitative nature of the changes in calcium concentration of various tissues during aging (Korenchevsky, 1961). Several factors probably contribute to the discrepancies in the literature regarding age-related changes in tissue calcium content: Different methods of calcium analysis, the non-uniform presentation of data, and variations in the maintenance of the experimental animals investigated may account for the discrepancies in the calcium values reported.

Aim of This Study

The present investigation is designed to study the calcium levels of various soft tissues of the laboratory rat at progressive periods throughout the life span. Two strains of highly inbred rats having differing average life spans are employed for purposes of comparison.

The 4 basic questions which this study attempts to answer are listed below and each is followed by a statement pertaining to its rationale.

Aim of the Within Strain Study

(1) Do certain soft tissues in extra-skeletal loci of the mammalian body show an accumulation of calcium with increasing age?

Sherman and MacLeod (1925) have reported that the whole body calcium content of the laboratory rat does not reach a plateau until about 8 months of age; it has been shown, however, that the plateau for calcification of bone in the rat is attained at an earlier age (McBroom, Cornelison and Weiss, 1966). It appears, therefore, that some of the increase in the calcium content which occurs in the intact animal must be accounted for by sites in extra-skeletal tissue.

Selye (1962) has proposed that calcium has a particular affinity for tissue loci which have undergone trauma or cell death. Since some tissues, e.g., brain, cannot reconstitute themselves following cellular loss or impairment, such tissues would be prime targets for calcium deposition if Selye's theory is correct.

(2) Do some soft tissues exhibit a decline or, perhaps, remain unchanged with regard to their calcium content with advancing age?

Lengemann (1959) has reported that the calcium retention in many soft tissues is higher in old animals than in young ones. However, if Selye's hypothesis--that tissues with the capacity for self-regeneration are less subject to calcium accumulation--is correct, then some of the tissues studied by Lengemann, e.g., liver, could be expected to resist calcium accumulation because of that tissue's capacity for self-

maintenance. It would seem that Selye's theory, while having a certain degree of applicability, is probably not the sole determining factor in the changing calcium content of various aging tissues, since tissues having a comparable capacity for self-maintenance may exhibit differing age-related calcium responses in different organisms (Korenchevsky, 1961).

Aim of the Between Strains Study

(3) Do tissue calcium alterations occur at ages which are chronologically dissimilar in rats having differing average life span?

(4) Are calcium concentrations in the same tissues comparable in the two rat strains at ages which represent equivalent percentages of the respective total life expectancies?

If calcium alterations are progressive changes which occur as a function of the stage of the animal's life span, such alterations should be chronologically dissimilar. They should, however, occur at roughly the same stage of life in rats of different average life spans. Because of the heterogeneous methodology, it would be meaningless to compare calcium changes in different rat strains by integrating the findings in the existing literature. Since no study has been performed which lends itself to comparisons between strains that were maintained simultaneously and in an identical manner, or in which the tissue calcium levels were measured by the same technique, such an investigation is needed.

Processes Associated with Aging

A General Discussion

Krohn (1966) believes that there is probably no single event which can be termed the aging process, but that a series of interrelated

processes are at play. The view proposed by Reichel (1966) is that aging represents a progressive loss of physiological capacity or function and that the processes involved result in an increased probability of death within a given period of time. Bertolini (1964) agreed that a decrease in physiological capacity is a consequence of aging, but emphasized that for the most part the etiology of the processes involved is unknown.

Aging may be viewed as a reduction in the reserve capacity of an organism, i.e., the capacity to cope effectively with a disturbance in the equilibrium (Shock, 1962; Samis, 1966; Calloway and Kujak, 1966; Lansing, 1947b, 1951). The causes of this reduction in physiological capacity have been the subject of some disagreement, but the basic view of Lowry et al. (1946) that aging is not a uniform process in all cells and organs has been shown to have some merit.

Two major causes of the time-related reduction of physiological capacity have been proposed: (1) The physiological capacity is reduced by an ever-increasing loss of cellular units from various organs and tissues. Shock (1962) believes that the body "dies" a little every day through the loss of functional cells. Lowry et al. (1946) expressed the view that aging is probably the effect of a loss of cells and alterations in tissue architecture, rather than a decrease in the capacity of cells to perform their function. (2) Curtis (1964) is a major proponent of the idea that individual cells age and lose the ability to resynthesize themselves, but that a tissue whose cells continue to divide actively could, at least theoretically, maintain a semblance of immortality. Another proponent of this idea (Reichel, 1966) believes

that among the multiple causes of aging, an accumulation of changes, mutations, or local chemical damage incapacitates individual cells and eventually causes a decline in the physiological capacity. Some earlier workers also held with the latter theory. For example, Lansing (1947b) and Heilbrunn (1952) expressed the view that in the last analysis senescence is a result of protoplasmic changes which occur in individual cells. Both of the above proposals are probably involved in the aging of the organism as an integrated whole, i.e., various tissues reacting differently to the passage of time.

Destructive effects of the environment and genetic factors can also be implicated in the reduced functional capacity which is often seen in old age (Shock, 1967). The effect of the genetic, clock-like factor has been stressed by the contention of Calloway (1966a) that each individual organism, if placed in a perfect environment, would still experience senescence and would die at a predetermined time. The practicality of this contention is very limited because seldom, if ever, is an organism presented an opportunity to actually live in a perfect environment. Rather, the organism is continually subjected to stresses, e.g., disease processes, and an increased susceptibility to certain diseases is one of the concomitants of aging. The major disease processes encountered by senescent mammals seem to be fairly universal. The most frequently occurring diseases which accompany aging in both man (Kirschenfeld, 1967) and the rat (Berg and Harmison, 1957) include the following: failure of the cardiovascular, pulmonary and/or renal systems; metabolic and/or neuro-muscular disorders; and, neoplasm formation. Many of these diseases have recently received much emphasis in the study of auto-immune processes which may be related to aging.

Effects of Age on Whole Body Composition

Changes in the composition of the mammalian body with growth and aging have been the basis for much study during the past six or seven decades. One of the first major efforts of this kind was reported by Lowrey (1913) who studied the contribution made by various organ systems to the total body weight of the rat from birth to one year of age. His findings show that the skin, skeleton and viscera contribute a lower percentage to the total body weight at one year of age than at birth, while muscle contributes a greater percentage at one year of age than at birth.

In 1917, Hatai performed an inclusive study of the compositional changes of the whole body in aging rats. He found that the greatest changes in body composition, as well as in external appearance, occur during the first three weeks of life--the period during which the diet consisted entirely of milk. Comparable findings were reported by Moulton (1923) who proposed that chemical maturity (a term coined by Moulton) is reached at about 50 days of age in the rat.

Hatai (1917) reported that virtually all of the change in the percentage of dry substance during the first year of life in the rat is accomplished during the first three weeks after birth. Hatai also observed that chemical changes during the early period of life appear to correspond chronologically with alterations in body water content. Moulton (1923) studied many mammalian species in addition to the rat and maintains that, although various mammalian species attain chemical maturity at different ages, these ages represent similar percentages of the respective life expectancies, e.g., man reaches chemical maturity at about 4.4 per cent and the rat at about 4.5 per cent of the expected

life span.

Spray and Widdowson (1950), who studied chemical composition changes throughout life, pointed out that attainment of chemical maturity does not imply complete absence of subsequent chemical alterations. Spray and Widdowson also believe that, although the body compositions of adults of different species are similar, the differences observed are probably as important as the similarities.

Several workers have studied aging changes in the composition of carcasses of mammals. A brief description of the pertinent findings is presented below: The percentage of body fat increases during growth and early adulthood and then falls in old age, but remains above the percentage present at birth (Hatai, 1917; Forbes, 1962; Spray and Widdowson, 1950). An increase in the percentage of organic material other than fat has been reported to occur during growth and aging (Moulton, 1923; Stare, 1967a), whereas the percentage of total body water has been reported to decrease during aging (Moulton, 1923; Forbes, 1962; Spray and Widdowson, 1950; Ruol, Menozzi and Furlanello, 1964). The percentage of total mineral content has been reported to rise during growth and aging (Hatai, 1917; Moulton, 1923; Spray and Widdowson, 1950; Cheek and West, 1956). Hatai has shown, however, that if the bone mineral is excluded from the measurements, the percentage of the remaining mineral declines with aging. This finding has found application in other studies involving various whole body analyses because in many instances bone tissue changes may oppose those seen in soft tissues, and even different soft tissues may change diametrically.

Effects of Age on the Composition of Various Tissues

Yannett and Darrow (1938) stated that the chemical composition of a tissue may be altered by either or both of the following mechanisms: (1) An increase in the size of the tissue mass by the addition of components or tissue cells whose composition differs from that of the original tissue; (2) Changes in the composition of the existing tissue.

In mammals, the percentage of water in all soft tissues which have been investigated falls from birth to mid-adulthood (Lowrey, 1913; Yannett and Darrow, 1938; Dulce and Guenter, 1961; Calloway, 1966b; Calloway and Kujak, 1966; Valcana, Vernadakis and Timiras, 1966). These investigators agree that the more rapid changes occur during early life.

Calloway (1966b) and Calloway and Kujak (1966) have found that a rapid decline in the percentage of ash, and a gentler and profound rise in organic material, accompany the decline in water content in all soft tissues which were studied.

In contrast to the above studies which were performed during the rapid growth stages of the animals and terminated before true old age was reached, Weller (1956) carried out a study on rats which was begun when the animals were one month old and continued until they had attained ages which represented true senescence. The findings indicated an increase in the percentage of water in the kidney, and decreases in the other tissues. With the exception of skin, however, none of the changes were statistically significant. These relatively small changes further support the idea that if major changes in tissue water content do occur, they must take place before the age of 1 month.

Manery and Hastings (1939) observed that while the volume of the

extracellular compartments varies from one tissue to another, the electrolyte concentrations in the extracellular fluids of all tissues are essentially identical.

Lowry et al. (1942) have reported that aging is accompanied by demonstrable changes in the proportion of extracellular and intracellular components of certain tissues, the most marked of these changes occurring early and late in life with little change during the intervening period.

The concensus among workers who have studied fluid compartment changes in relation to aging seems to be that the percentage of water in the extracellular compartments appears to decrease during growth, while the intracellular compartments remain essentially unaffected (Heppel, 1939; Yannett and Darrow, 1938; Andrew, et al. 1959; Irvine, Farrelly and Frazer, 1964; Vernadakis and Woodbury, 1964). On the other hand, the findings during senescence indicate that the percentage of water in the extracellular compartments increases, that in the intracellular compartments decreases, and the total percentage of water in the various tissues remains essentially unchanged (Andrew, et al., 1959; Mori and Duruisseau, 1960; Dulce and Guenter, 1961; Friedman, Sreter and Friedman, 1963; Friedman, et al., 1966). These findings imply that growth of tissues is accompanied by an absolute loss of water, while aging results in a shift of water from one compartment to the other with no change in absolute volume.

Effects on the Physical Properties of Cells

The electrolyte composition of tissues is thought to be dependent upon complex macromolecular states with which water is associated (Joseph, 1966). Joseph believes that the variable properties of cells during

growth are partially dependent on the states of aggregation of intracellular water, and that in senescent cells, chemical cross-linking may occur which decreases the dielectric constant and increases the ion-binding capacity of such cells. Such a cross-linking is commonly encountered in the collagenous component of many tissues during aging, (Milch, 1965).

Calcium

A General Discussion

Calcium ranks fifth in abundance of all the elements of the mammalian body and is the number one structural element. The major portion of calcium in the body occurs in the inorganic form (Comar and Bronner, 1964) and, since it tends to form insoluble compounds, it is understandable that it may accumulate in tissues with the passage of time (Lansing, 1951).

Rapid calcification in the tissues of an animal during growth--induced by diets containing high levels of calcium--resulted in an extension of the period between the attainment of maturation characteristics and the appearance of the signs which were associated with senescence (Sherman and Booher, 1931). On the other hand, rats which received a low calcium diet appeared normal, even though chemical analysis revealed their bodies to be calcium poor (Sherman and Booher, 1931).

The role of calcium in essential body processes, e.g., muscle excitation and contraction, blood clotting, etc., is well known. Calcium also plays a role in certain enzyme systems, in cell division, and in cell permeability. The regulatory function of calcium with regard to

ion flux at the cell membrane is reported by Comar and Bronner (1964), and Manery (1954) has implicated calcium in membrane hardening and intercellular cementing. Hickie and Kalant (1966) reported that the cells of rat livers which had been perfused with a solution containing ethylenediaminetetracetic acid (EDTA) became partially, and in some instances completely, detached from one another. This effect of EDTA, a calcium chelating agent, was attributed to the removal of calcium from cell surfaces and from intercellular loci.

Of the total body calcium about 99 per cent is located in the skeleton in both man (Henry and Kon, 1947) and the rat (Sherman and MacLeod, 1925). Much of the calcium in soft tissues seems to be loosely bound at the cell surface (Hickie and Kalant, 1966) and is probably associated with the ribonucleoprotein complex (Lansing, 1951; Manery, 1954). Manery (1954) has reported that calcium is present in fairly low concentrations in the cellular fraction of most soft tissues, but that the calcium concentration in skeletal muscle increases in certain pathological and nutritional conditions, such as muscular dystrophy, denervation atrophy, and Vitamin C deficiency, and during aging.

Changes During Aging

Calcium is a cation which is frequently associated with aging processes. Calcium content of the whole body of man (Forbes, 1962) and the rat (Sherman and MacLeod, 1925; Sherman and Booher, 1931; Weiss *et al.*, 1961) has been reported to increase with increasing age. The rise of calcium content in the carcasses of the rats observed by Sherman and coworkers was very rapid up to about 90 days of age, more gradual up to the age of eight months, and very slight thereafter. This slight increase

after eight months of age may occur in the soft tissues.

Sherman and MacLeod (1925) have observed that the calcium content of virgin female rats is higher throughout life than that of males, but the calcium level in females which have produced and raised offspring more closely corresponds to the level seen in males. Deuel et al. (1944) have also reported comparable sex differences in the whole body calcium content of rats. Spray and Widdowson (1950) have demonstrated that the higher calcium concentration in non-breeder female rats is primarily a reflection of the bone calcium content, and Spray (1950) has assigned the majority of the calcium loss seen in breeder females to the decreased bone calcium which accompanies the bearing and suckling of young. Spray believes further that the chemical composition of rats which have produced offspring is not restored to the premating level until approximately 80 days after the end of gestation, and is never regained as long as lactation is allowed to persist. However, Strebel et al. (1966) have shown that calcium deposition-particularly in the arterial system-is promoted by the cessation of lactation.

Henry and Kon (1953) and McBroom, Cornelison and Weiss (1966) have shown that when an adult stage is attained in the rat, the animal no longer deposits calcium in its bones. Thereafter, as aging progresses, an increase in catabolic processes leads to a loss of bone salts (Shah, Krishnarao and Draper, 1967) and calcium tends to be deposited in tissues other than bone (Henry and Kon, 1947; Lansing, 1947b; Mori and Duruisseau, 1960).

In man, increases in calcium content with aging have been reported in arteries, kidney, heart (Lansing, 1951), brain and skeletal muscle

(Korenchevsky, 1961).

In the rat, increases in the calcium content have been reported in kidney, brain, lung, aorta, heart and skeletal muscle (Freydberg-Lucas and Verzar, 1957), but these workers used a titration method for calcium analysis which is subject to increased values as a result of interfering cations. Other workers, however, have reported decreases in the calcium contents of kidney and liver when expressed per unit of wet weight (Shah, Krishnarao and Draper, 1967), in both intracellular and extracellular fluids of cardiac muscle (Mori and Duruisseau, 1960), in the in vitro calcium retention in skeletal muscle (Weller, 1956), in small intestine (Freydberg-Lucas and Verzar, 1957), and in the plasma (Bekemeier and Hannig, 1960) of aging rats. Some of the findings cited above do not include statistical results and the changes reported may represent only trends which could prove to be insignificant. The frequent discrepancies in the above findings are quite evident and Korenchevsky (1961) has pointed out that discrepancies are also quite common in the data available for aging changes in calcium contents of the soft tissues of man. This disagreement may be a result of the various methods of analysis employed by the different investigators, as well as the different reference bases upon which the data are expressed.

Lansing (1947a, 1947b) stated that calcium changes in most aging tissues are probably related to aging changes in the lipoprotein-containing structure at the cell surface.

Methods of Measurement

Many methods have been devised for measuring the calcium concentration in biological materials. One of the methods which is still widely

used for quantitative calcium determination was developed by Clark and Collip (1925). The procedure involves the precipitation of calcium as an oxalate complex and its subsequent conversion to a permanganate complex which is titrated to an end-point for quantitation. This method, although quite accurate, has the disadvantages of being time-consuming, requiring a large quantity of sample, and exhibiting a fading end-point. Many modifications of the Clark-Collip method have been used effectively, but the disadvantages of the original method remain in varying degree (Catledge and Biggs, 1965; Connerty and Briggs, 1965; Kimsey, 1965; Bellinger and Campbell, 1966).

Flame photometry has been applied to calcium measurement in biological samples (Burriel-Marti and Ramirez-Munoz, 1957; Evans, Lind and Wiederanders, 1967), but this method is plagued by interference effects which can be eliminated by precipitation and resuspension of the calcium. This process, however, also proves to be quite troublesome and time-consuming.

Calcium determinations have been performed by fluorometric methods (Kepner and Hercules, 1963) in which the major problem is again one of interference by other substances encountered in biological samples.

Electrodes designed to measure calcium ion activity (Ross, 1967) provide a method for rapid calcium measurement. Here too, the problem of interference is encountered and slight variations in the pH of the sample affect the values obtained.

A method for calcium determination which has recently become popular is that of atomic absorption spectrophotometry. The discussion that follows presents a brief history of the development of the method

and the advantages it offers over other methods.

Atomic Absorption Spectrophotometry (AAS)

Historical Review

Wollasten, in 1802, was the first worker to report a phenomenon caused by atomic absorption when he observed dark bands in the sun's spectrum, but he was not aware of their cause. In 1814, Fraunhofer was able to map over 500 absorption lines in solar radiation (cited by Zettner, 1964).

A typical atomic absorption experiment was performed by Foucalt in 1849, in which he passed a ray of sunlight through a carbon arc and then through a prism to produce a spectrum. The expected dark bands were observed but they were more completely blacked out after having passed through the carbon arc. It was later realized that the "occult power" of the carbon arc was due to sodium contamination of the carbon electrodes and the resultant absorption of the sodium resonance lines of the solar spectrum (cited by Zettner, 1964).

The foundation for modern spectrochemical analysis was laid by Kirchoff and Bunsen (1860a, 1860b, 1861a, 1861b) who applied their knowledge of the nature of the Fraunhofer lines to analytical procedures.

AAS in its present form was developed in Australia by Walsh (1955) who recognized the advantages inherent in the use of absorption methods of analysis over those procedures which rely on emission. The advantages are that emission requires the ions to be in an excited state, while absorption is accomplished by atoms in the ground state. When a solution is aspirated through a flame, only about 5 per cent of the atoms

become excited and emit light. Thus, some 95 per cent of the atoms remain in the ground state and are capable of absorbing light at their respective resonance lines only. Willis (1960), also an Australian, is credited with the application of AAS to the determination of calcium in biological materials.

Why AAS Was Chosen for This Study

It has been reported that calcium measurement by AAS is superior to existing methods in regard to accuracy, precision, and ease and speed of performance (Zettner and Seligson, 1964; West, 1967).

According to Willis (1965), once a tissue has been ashed and the ash suspended in dilute acid, virtually the only limitation of the AAS method is the sensitivity of the system for the specific metal to be analyzed. Furthermore, the standard solution need contain little other than a known amount of the element being investigated in a solvent similar to that used for dissolving the sample ([Slavin], 1966).

With the exception of isotopic forms, the absorption lines of various elements do not overlap. Therefore, AAS is relatively free from spectral interference and can be used to determine elements in a matrix that is essentially unknown (Zettner, 1964; Willis, 1965; [Slavin], 1966; West, 1967), and the term "unknown matrix" exactly describes the tissue samples analyzed in the present study.

Flame temperature has little effect on AAS since the method requires that the atoms be in the ground state, and excitation interference, i.e., interference due to emitted light, appears to be entirely negligible because of the insignificant number of atoms which become excited in most flames (Zettner, 1964; Willis, 1965; [Slavin], 1966).

AAS is less subject to cationic and anionic interferences than is emission spectrophotometry (Willis, 1960) and competitive cations may be added to the sample to control depression by anions (Zettner, 1964).

Oxalate precipitation, a suitable reference procedure despite its technical difficulties (Trudeau and Frier, 1967), has been compared to AAS for calcium analyses and the differences noted were of no practical significance (Gimblet, Marney and Bonsnes, 1967).

In summary, the use of AAS for the determination of the calcium content of biological materials enables one to perform the analyses with minimal interfering effects from unknown quantitites of other constituents of the sample, and to do so with relative ease, precision and accuracy.

CHAPTER II

MATERIALS AND METHODS

Animals

The laboratory rat is an experimental subject well suited for the study of aging processes in mammals because it has a conveniently short life span, and because it is readily available.

The subjects for this study consisted of two strains of highly inbred rats. Both strains were developed by Dr. Maynie R. Curtis at Columbia University and both have been inbred through brother-by-sister matings since the 1920's. The Fischer strain was the first to be developed by Curtis, and the initial mating occurred on September 22, 1920. The A x C strain was developed from a mating between a rat of the August strain and a rat of the Copenhagen strain on December 15, 1926 (Dunning, 1957).

These rat strains are advantageous to this study for two reasons: (1) the uniform genetic make-up of inbred animals tends to reduce the normally occurring biological variation within each strain; and, (2) these two strains are known to have differing average life spans, a characteristic which makes them particularly suitable for a comparative study of aging changes.

The Fischer rats are the shorter-lived of the two strains and have an average life span of some 12 months. According to Curtis, Dunning and Bullock (1933), the average life span of the Fischer strain was 13.6

months in the early 1930's. The females were longer-lived than the males, i.e., 14.1 and 12.7 months respectively. After some 60 generations of inbreeding, the average life span of the males and females combined was 12.3 months (Dunning, 1957).

Curtis and coworkers reported that the average life span of rats of the A x C strain was 21.7 months in 1933. This figure remained unchanged after some 47 generations of inbreeding, and no significant sex difference was found among rats of the A x C strain (Dunning, 1957).

At present, colonies of both of these strains are maintained at the University of Oklahoma Medical Center. They are now approaching the one-hundredth generation of inbreeding and the average life spans of the two strains are similar to those noted by Dunning some 10 years ago.

Although the two rat strains differ genetically, they are maintained identically from an environmental standpoint. They are housed in the same room, cared for by the same attendants, and receive identical diets. The temperature is controlled at 21-24°C. The rats used for internal standards were kept in other quarters, but under similar conditions.

The rats are weaned at 4 weeks of age, and breeder rats are retired at the age of approximately one year. The animals used in this study were kept in groups of 5 to 8 rats per cage and received a standard diet of laboratory chow. The diets were supplied by Teklad Incorporated (Rockland Farms, Monmouth, Illinois) and Purina Company (St. Louis, Missouri). The diets from these two sources do not differ appreciably and their compositions are given in APPENDIX I.

Rats used in this study exhibit characteristic growth curves (Figure 1) showing an initial increase in body weight which is very rapid, followed by a markedly diminished rate of increase.

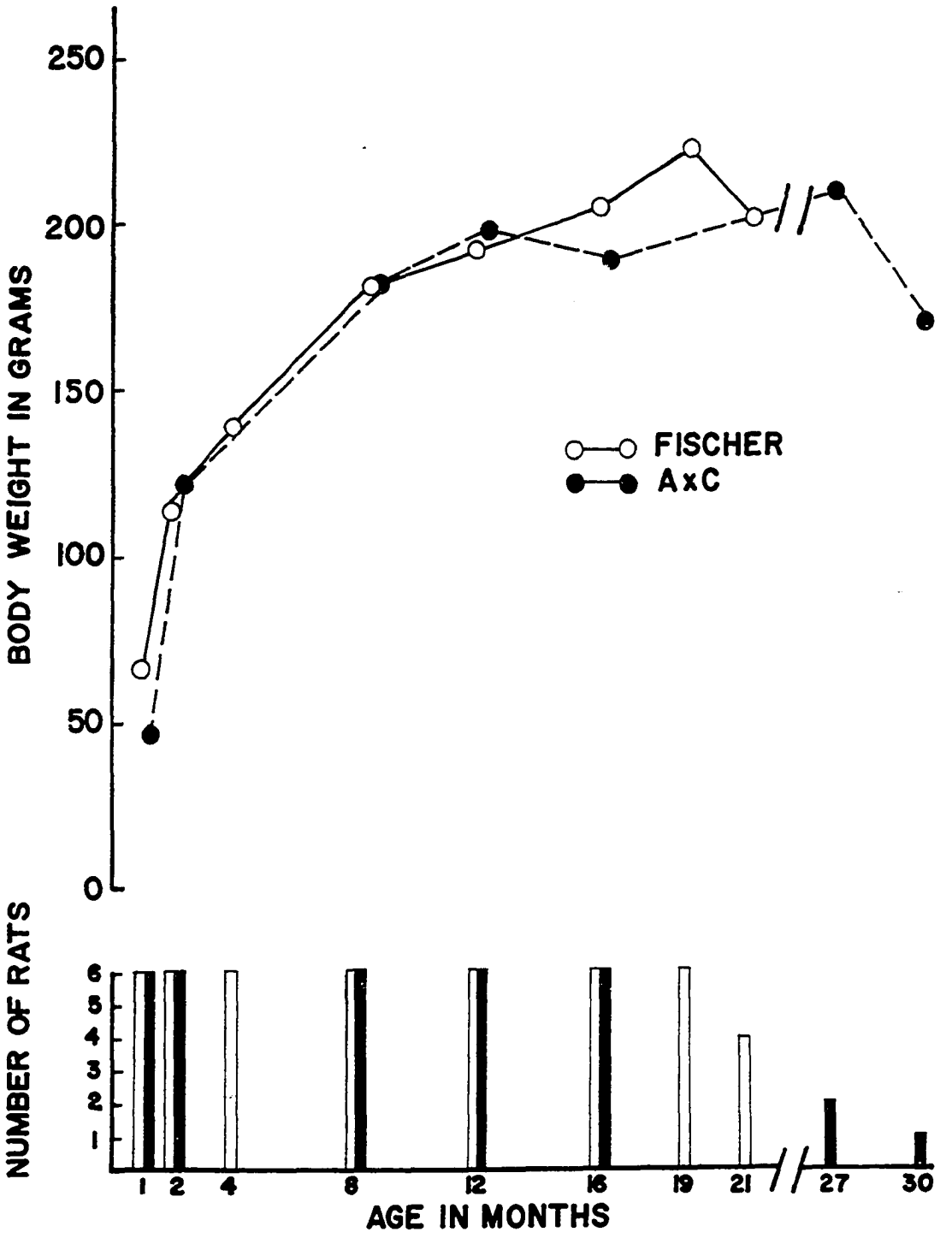


Figure 1. Mean body weight in grams of Fischer (○) and A x C (●) rats during the life span.

This study was conducted in two parts: (1) Forty-six Fischer and 21 A x C rats, all females, were used initially and, with a few exceptions, the animals were unmated. Groups of Fischer rats were selected at ages 1, 2, 4, 8, 12, 16, 19 and 21 months to provide data for a complete and sequential investigation. Fewer age groups were selected from the A x C strain because a smaller number of these animals was available. The following A x C age groups were selected for study: One, 2, 16, and 27-30 months. The data from these A x C animals were utilized primarily for the comparative aspect of the study. The older age groups in each strain contained smaller numbers of animals than the younger age groups due to the high mortality rate among old animals. (2) Additional data were collected subsequently in an effort to clarify and substantiate certain findings suggested by the original sets of data. These additional data were composed of the following groups of animals: Twenty-six Fischer rats in age groups of 1, 12, 16 and 19 months, and 11 A x C rats in age groups of 2 and 22 months. Each of these age groups consisted of 5-7 rats depending on the number of animals available.

These two parts of the study were considered both independently and in a combined fashion. Essentially only the combined data are presented in Chapter III, but the individual parts of the study are described separately where discrepancies appear to exist between the two sets of data. It is known that death in old rats is preceded by a period of body weight loss (Yiengst, Barrows and Shock, 1959; Berg, 1965). As shown in Figure 1, the oldest age groups used in this study exhibit the weight loss phenomenon and, on the basis of this criterion, can be regarded as truly old.

Methods

Preparation of Experimental Animals

Each experimental animal was caged singly on the day prior to sacrifice and was deprived of food for 16-20 hours before the experimental procedure was begun, so the rat would be in a post-absorptive state (White and Rolf, 1955). Drinking water was removed one hour before the experiment was begun.

The animals were anesthetized with ether and the ventral surface was shaved with small-animal clippers to facilitate the subsequent removal of a skin sample. An opening was made in the abdominal wall and the distal regions of the abdominal aorta and inferior vena cava were exposed. Both vessels were cannulated with the cannulae being directed centrally. The venous cannula was occluded and a blood sample was obtained from the aortic cannula, as described by Streicher (1959) and Vernadakis and Woodbury (1964). Approximately 3 ml. of blood were collected in a centrifuge tube containing a drop of sodium heparinate compound (Anti-Clot, Clinton Laboratories, Los Angeles, California). One ml. of the blood sample was removed for whole blood analysis and the remaining 2 ml. were centrifuged to provide a plasma sample from which a 0.50 ml. aliquot was taken for analysis.

Widdowson and Southgate (1959) have shown that a rat exsanguinated by sectioning of the abdominal aorta, or by decapitation, retains some two-thirds of its total blood volume. In an attempt to remove as much of the remaining blood as possible from the tissues, the animals used in this study were perfused with isotonic sucrose (0.25M) (Umbreit, Burris and Stauffer, 1964), and the pH of the solution was within the 7.48-7.52

pH range reported by Reed, Withrow and Woodbury (1967) for plasma obtained from the abdominal aorta of an ether-anesthetized rat.

The perfusion fluid was administered via the venous cannula and the perfusion rate was kept as constant as possible. The perfusion procedure was deemed complete when the fluid collected from the aortic cannula became colorless, an end point which required 30 ml. of perfusion fluid, or less, depending on the size of the animal being perfused. When the perfusion was complete, the lungs and cerebral cortex had a white, bloodless appearance, the liver, kidneys and heart were a light salmon color, and the gastrointestinal viscera appeared blanched. The time elapsed during the procedure was seldom more than five minutes.

In order to evaluate the effect of the perfusion on the calcium contents of the different organs, calcium values obtained from organs of perfused animals were compared with those from animals which had been sacrificed by decapitation. Both groups of animals were of the same age, sex and strain, and the results are presented in Table 1.

Manery (1954) has shown that blood which remains in tissues following sacrifice has a negligible effect on electrolyte measurements, and this finding is confirmed by the results in Table 1 in respect to the measurement of tissue calcium content. In addition, Hickie and Kalant (1966) have shown that no statistically significant difference occurred between calcium contents of 5 rat livers which have been perfused with Krebs-Ringer phosphate solution and 30 which have not been perfused.

Widdowson and Southgate (1959) have shown that skeletal muscle is the most susceptible tissue in the rat to electrolyte loss during severe hemorrhage. The skeletal muscle selected for analysis in this study,

TABLE 1

EFFECT OF PERFUSION WITH ISOTONIC SUCROSE ON TISSUE CALCIUM CONTENT[a]

Tissue	n	Perfused Tissue Ca	n	Non-perfused Tissue Ca	p value[b]
Kidney	5	17.08 ± 0.81	6	15.61 ± 0.47	n.s.[c]
Lung	6	23.37 ± 1.75	6	26.06 ± 1.75	n.s.
Cerebral cortex	6	11.79 ± 0.40	6	11.96 ± 0.45	n.s.
Liver	6	7.96 ± 0.55	5	6.72 ± 0.58	n.s.
Skin	6	15.81 ± 1.37	6	17.30 ± 1.18	n.s.
Cardiac muscle	6	13.49 ± 1.43	6	13.34 ± 0.82	n.s.
Aorta	5	102.79 ± 12.76	8	78.37 ± 3.86	n.s.
Intestinal smooth muscle	6	24.68 ± 2.96	8	30.97 ± 2.68	n.s.

[a]Calcium content expressed as mean value ± standard error in mEq/kg dry tissue weight.

[b]p value for 0.05 level of significance was obtained by a weighted Student's 't' test analysis using the formula

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\bar{s}_d}$$

where $\bar{s}_d$ is the weighted standard deviation of means.

[c]n.s. = no significant difference at the 0.05 level.

i.e., gastrocnemius, was excluded from the blood removal system, and consequently, also excluded from the perfusion circuit.

Preparation of Tissue Samples for Calcium Analysis

The tissues selected for study were removed from the animal as quickly as possible following the perfusion procedure, cleaned of any visible extrinsic tissue, and placed into previously tared, fused silica crucibles (Vitreosil, Arthur H. Thomas Company, Philadelphia, Pennsylvania) for subsequent preparation. Plasma and whole blood samples which were obtained were also placed into crucibles. All tissues were handled with clean forceps to avoid contamination.

In addition to plasma and whole blood, samples taken from liver, kidney, lung, cardiac muscle (ventricular), cerebral cortex, skeletal muscle (gastrocnemius), intestinal smooth muscle (jejuno-ileal region), and skin were analyzed for calcium content.

The tissue samples were dried to constant weight (24 hours) in an electric oven at 100-110°C (Flear and Florence, 1961; Law and Phelps, 1967). The samples were then removed from the oven, allowed to cool for 10 minutes, and weighed on a Mettler analytical balance. Because inconsistent results have been reported for age-related tissue water content (page 8), and since some of the tissues retained fluid from the perfusion procedure, the weight obtained following the drying period was the reference base upon which the calcium values were expressed.

The dried tissue samples were placed into a cold muffle furnace and ashed by increasing the temperature to 500°C over a period of five to six hours. The temperature was maintained at that level for the

ensuing 36 hours at which time ashing was complete (Harrison, 1953; Lifshitz et al., 1967). The plasma was ashed along with the other tissues as described by Heppel (1939).

Grove, Jones and Mathews (1961) have observed that 550°C is the maximum temperature which can be maintained for 24 hours without the loss of sodium and potassium from the sample, although no loss of calcium could be demonstrated at temperatures under 700°C. Grove and co-workers also contend that an ashing period of more than 24 hours at 400-500°C results in no apparent loss of sodium and potassium; and, in samples weighing under 5 grams, most of the carbon is burned from the ash at 450-500°C in 24 hours. However, plasma requires either a longer ashing period or a higher temperature. Since this study includes plasma, the ashing time was extended to 36 hours. Although 550°C is the temperature below which no electrolyte losses are believed to occur, the temperature chosen for this study was a thermocouple reading of 500°C. Hamilton, Minsky and Cleary (1967) have recently shown that the actual temperature in different parts of a muffle furnace may vary as much as 50°C from the temperature recorded by the thermocouple.

At the end of the 36 hour ashing period, the samples were removed from the muffle furnace, covered and allowed to cool for 30 minutes, and weighed on an analytical balance. The ash weights obtained were expressed as a percentage of the previously recorded dry weights. The relationship of the ash weight to age has been studied, but is not reported in the present work.

After the ashed samples had been weighed, the residue was dissolved in a few drops of concentrated nitric acid and heated to dryness on a

hot plate. A few drops of perchloric acid (70 per cent) were then added and the sample was again fumed to dryness by heating. The colorless, crystalline material which remained in the crucible was resuspended in 1.0 N hydrochloric acid (Grove, Jones and Mathews, 1961; Marcus and Wasserman, 1966) and quantitatively transferred to volumetric flasks. Following the quantitative transfer, the samples were brought to the desired volume with 1.5 per cent (w/v) lanthanum chloride (K and K Chemical Company, Plainview, New York) in deionized water. The samples collected for the second part of this study were prepared to the stage just described and stored in chemically inert plastic vials and analyzed after the entire sample collection was completed because the analytical equipment was not immediately at hand. The final concentration of hydrochloric acid in the sample was 0.10 N to 0.12 N (v/v), and the final lanthanum chloride concentration was 1.0 to 1.3 per cent (w/v). These final concentrations are in good agreement with those recently reported by Klein, Kaufman and Morganstern (1967) and Hickie and Kalant (1966) for hydrochloric acid and lanthanum chloride respectively. The rationale for the use of lanthanum chloride in the samples is discussed in the following section.

Tipton et al. (1963) believe that dry ashing is the most satisfactory method for preparing tissues which are to be analyzed for a large number of elements. Burck (1961) has reported that although dry ashing is slow and extensive extraction procedures are required, the results are good.

A wet ashing procedure which utilized sulfuric acid (Gettler and Weiss, 1943) was tested for use in this study, but as reported by Middleton and Stuckey (1954), the presence of sulfuric acid caused the formation

of highly resistent compounds which prevented the accurate determination of calcium concentrations in the samples. Dry ashing as described above was therefore selected as the method of choice for the present study, and to date is still the most acceptable method for preparation of tissues from which calcium determinations are to be performed (Bradbury et al., 1968).

Calcium Analysis

The instruments used were obtained from Beckman Instruments, Inc. (Fullerton, California) and consisted of the following components: A Model 1403, DB-G spectrophotometer with a Model 315800 potentiometric strip chart recorder; a Model 1301 atomic absorption unit with a hollow cathode lamp as the light source; and, a turbulent flow aspiration system which employs a series of 3 total consumption burners. Kahn (1966a) has stated that the total consumption burner is superior to the other available type--the laminar flow burner--with respect to response time, low "memory" effect, ease of maintenance, and the large number of oxidants which may be used.

The fuel and oxidant employed by this system are hydrogen and air respectively, in a mixture which produces a flame temperature ranging from 2045 to 2127°C (West, 1967; Willis, 1960).

The hollow cathode lamp used is specific for the spectral reasonance line of calcium and emits light of that order only. A typical record of the emission spectrum of the calcium lamp (Figure 2) at wavelengths in the vicinity of the calcium resonance line illustrates the specificity of the spectral band produced at a wavelength of 423 millimicrons.

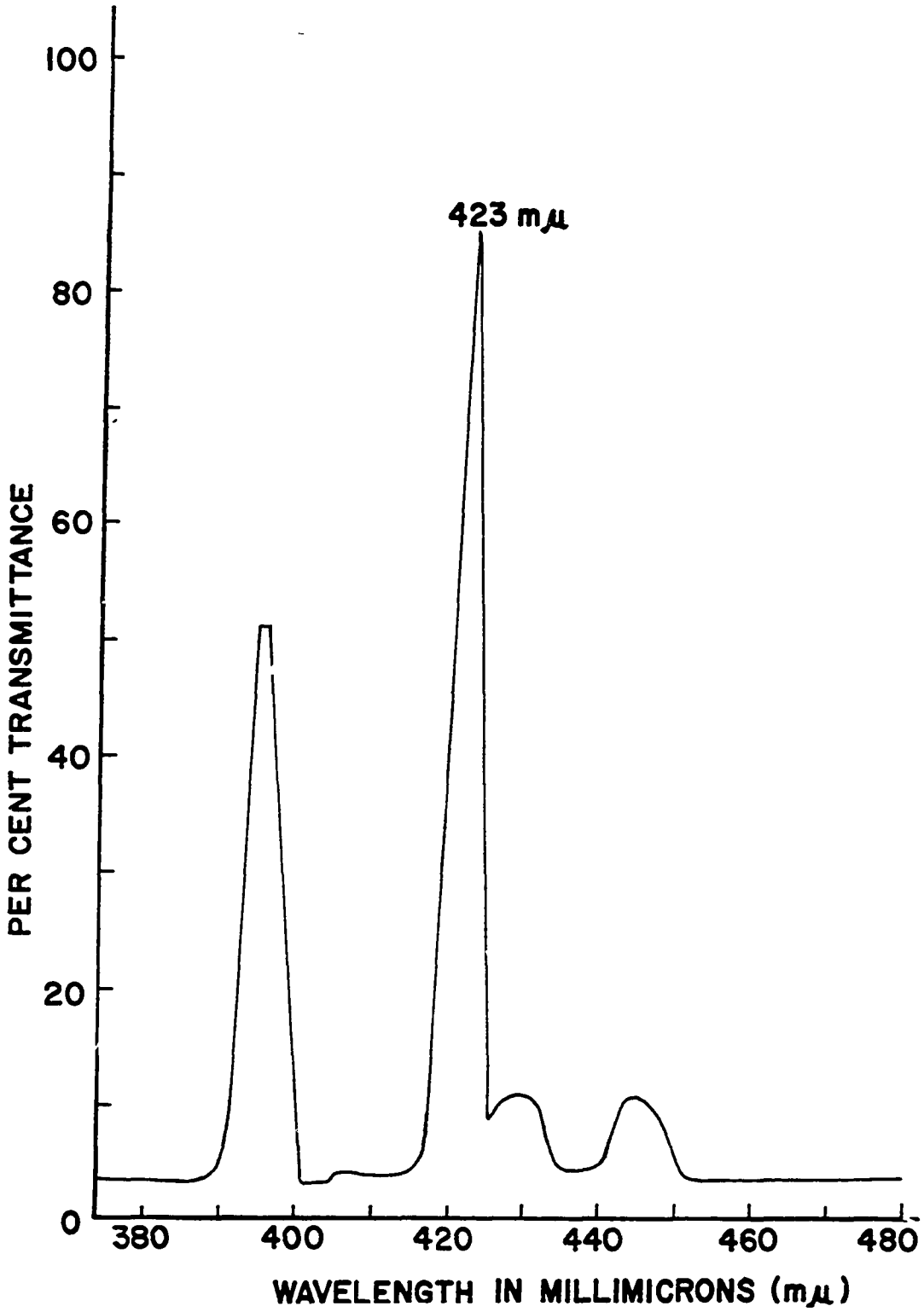


Figure 2. Emission spectrum of the calcium hollow cathode lamp.

In order to prevent undesired flame radiation from interfering with the measurements obtained, the light source is modulated before entering the flame. The amplifier system is of the a.c. type and is adjusted to respond only to the modulated frequency of the lamp. According to Zettner (1964), the contribution of the flame to the measured signal in such a system is negligible in practical use.

The settings of the instrument system described above are given in Table 2 as they apply to the determination of calcium concentration in tissue samples. References are included when the parameters used are based on those recommended by other investigators.

Before the calcium determinations could be performed, it was necessary to obtain certain information about the measuring system such as: The linear concentration range for plotting a usable standard curve; the sensitivity of the system; and, the effects of various substances which tend to cause interference, and how the interference can be controlled. The discussion which follows describes experiments which were performed to provide information regarding these points.

A large range of calcium concentrations were analyzed and the values obtained were plotted on a semilogarithmic scale to determine the linear range (APPENDIX II, page 118). The calcium concentration range which provides a linear plot was found to be 0 to 1.0 mEq/l (0-20 ppm).

The sensitivity, or detectability limit, i.e., that concentration of calcium which gives a reading equal to one percent of the usable concentration range, was found to be less than 0.025 mEq/l (< 0.5 ppm). This can be determined from APPENDIX II (page 119) where it is seen that the linear concentration range of 0 to 1.0 mEq/l represents readings of 100 to 8 per cent transmittance, a range of 92 per cent transmittance.

TABLE 2

INSTRUMENT SETTINGS FOR DETERMINATION OF CALCIUM

Parameter	Optimum Setting	References
Wavelength	423 millimicrons	a,b
Slit width	0.2 mm	b,c
Number of light Passes	3	d
Number of burners	3	d
Distance of burner tips below light path	0.4 inches	d,e
Air pressure	18 lb/sq. in.	f
Fuel pressure	7 lb/sq. in.	f
Hollow cathode lamp current	10 milliamperes	f
Sample aspiration rate	5.0 ml/min./burner	f

[a]Decker, Aras and Decker (1964).

[b]Manning and Capacho-Delgado (1966).

[c]Kahn (1966b).

[d]Zettner (1964).

[e]Willis (1960).

[f]Determined in our laboratory from permissible ranges recommended by the manufacturer.

One per cent of this range is 0.92 per cent transmittance, and the plot shows that a calcium concentration of 0.025 mEq/l gives a deflection of some 4 per cent transmittance, or far more than the minimum required by the definition of the detectability limit. Comparable detectability limits have been reported by Willis (1965) and Zettner (1964).

Anions, as well as other cations, can affect calcium measurements by atomic absorption spectrophotometry. Phosphorus is the most notorious interfering anion encountered in the analysis of biological materials for calcium content. However, this effect can be overcome by the addition of a competitive cation, such as lanthanum or strontium, which combines preferentially with the phosphorus and prevents the formation of calcium phosphate compounds (Monder and Sells, 1967; Trudeau and Frier, 1967; Willis, 1960, 1961, 1965; Zettner, 1964).

Potassium and magnesium are reported to have no appreciable effect on the determination of calcium by atomic absorption spectrophotometry, even when they are present in concentrations that exceed those normally present in biological material (Willis, 1960). Sodium, however, has a slight depressive effect which can be minimized by the addition of lanthanum chloride to the sample solution (Trent and Slavin, 1965; Zettner and Seligson, 1964).

The capacity of a lanthanum chloride diluent to minimize interference effects was tested on our instrument system by the analysis of calcium alone, in the presence of various concentrations of other elements in a water diluent, and in the presence of the same concentrations of other elements in a lanthanum-containing solution. The results obtained (APPENDIX II, page 120) indicate that the addition of lanthanum

chloride virtually eliminates the interference seen when a pure aqueous diluent is utilized.

Widdowson and Southgate (1959) have observed that electrolyte values obtained by photometric analysis of acid-containing tissue extracts differ by as much as 40 per cent from the values obtained by chemical methods.

The effects of various acids and various normalities of acid on the calcium values obtained were tested. The results are presented in APPENDIX II (page 121). The findings are in agreement with those of other recent reports (Monder and Sells, 1967; Hanig and Aprison, 1967), which have shown that low concentrations of nitric acid have a depressive effect on calcium values obtained, while the presence of dilute hydrochloric acid has an enhancing effect. The findings of the present study indicate that low concentrations of sulfuric acid have a greater depressive effect than those of dilute nitric acid, and the presence of sulfuric acid results in the virtual extinction of the calcium-measuring capability of the system.

The standard solutions were prepared from a stock calcium solution (Harleco Corporation, Philadelphia, Pennsylvania) containing 100 mEq/l, and the standards contained lanthanum chloride and hydrochloric acid in the same concentration ranges as those of the samples (page 27). Both the standard solutions and the diluent were stored in polyethylene bottles when kept for more than a few hours, as recommended by Willis (1960).

The following experiment was performed to determine the amount of the total calcium which can be recovered from a sample after its having

been subjected to the experimental procedures used in this study: A solution containing a known amount of calcium (0.400 mEq/l), along with magnesium, sodium and potassium was prepared and analyzed for calcium content. An aliquot of the sample was then subjected to drying, ashing, acid extraction, resuspension, and quantitative transfer as described earlier in this chapter. The sample was diluted appropriately in the lanthanum chloride diluent and again analyzed for calcium content. The results of a typical experiment are presented in Table 3.

From these data it is reasonable to conclude that calcium recovery is complete within the limits of error of the measuring system, and the minute calcium loss observed lies well within the recovery limits reported by other investigators (Bradbury et al., 1968).

TABLE 3

CALCIUM RECOVERY FROM A SAMPLE OF KNOWN CALCIUM CONCENTRATION

Sample Treatment	Transmittance (%)	Calcium Concentration[a] (mEq/l)	Recovery (%)
None	39.5	0.400	100.0
Complete experimental procedure	40.0	0.398	99.5

[a]Calcium concentration obtained from standard curve in APPENDIX II (page 122).

Presentation and Analysis of Data

The values obtained in this study are expressed in milliequivalents of calcium per liter (mEq/l) of plasma or of whole blood, and in milliequivalents of calcium per kilogram of dry tissue (mEq/kg) for the

soft tissues. Dry weight was chosen as the reference in order to avoid any effects which varying amounts of tissue hydration could exert on the results obtained. Sample calculations are presented in APPENDIX III (page 124).

Two types of statistical tests were used to analyze the data. A within strains comparison was made within each strain of rats. This analysis was applied to compare the calcium content of each tissue in a given age group with that of the same tissue in every other age group. The testing procedure employed for this comparison was the New Multiple Range Test as devised by Duncan (1955) and modified by Kramer (1956). This test was performed at the 0.01 level of significance and a sample analysis is presented in APPENDIX III (pages 125-128). Both the original data and the combined sets of data, i.e., the original and that collected subsequently, were subjected to the same statistical treatment. Where points of apparent discrepancy are indicated between the results obtained from the two sets of data, a detailed description of the findings accompanies the presentation of the results.

Comparisons of the calcium contents were made between the two rat strains and the analysis employed was the weighted Students 't' test used earlier (page 24). These analyses were applied to the calcium contents of the same tissues in the two strains at ages which are regarded as physiologically equivalent, i.e., at ages which represent comparable percentages of the respective life spans. Three comparisons of this type were performed for each tissue at the following ages: One-month-old Fischers and one-month-old A x C rats (representative of less than 5 per cent of the respective life spans); Fischer rats of 12 months and A x C

rats of 16 months (representative of 50-55 per cent of the respective life spans); and, 21-month old Fischer rats were compared to 27-30 month old A x C rats (age groups which represent 90-100 per cent of the respective total life spans).

CHAPTER III

RESULTS

The results presented in this section are described in terms of both chronological age and physiological age ranges of the subjects. When reference is made to chronological age, the parameter used is simply the age expressed in months. The phrase 'physiological age' refers to an arbitrary parameter used to express the age of the animal as a percentage of the maximum total life span of the strain which it represents. The physiological age ranges chosen for comparison represent young, middle-age and old animals of the two rat strains.

The results of the study are presented in the following order: (1) the within strains, i.e., longitudinal, age-related findings of tissue calcium concentration in each rat strain; (2) the between strains, i.e., comparative findings from the standpoint of chronological age, physiological age range, and in relation to the overall mean calcium levels throughout life in each tissue investigated; and, (3) a summary of the major results obtained.

Findings Within the Strains

The results are presented in graphs showing the calcium concentrations of a given tissue at progressive age levels in both rat strains. The upper portion of each figure shows the calcium values obtained, and

the lower portion shows the number of rats represented by each of the corresponding mean calcium values. A table depicting the results of the statistical analyses performed on the corresponding tissue calcium values in each strain during aging is located immediately after each figure.

The results presented represent the data obtained by the combination of two separate sets of experiments. The initial data which were collected (hereafter referred to as original data) were subsequently combined with data collected at a later time (internal standards). These internal standard data were collected in an attempt to provide clarification and additional support for the findings suggested by the original portion of the study. The samples which comprise the internal standard were handled identically to those of the original samples insofar as was possible, with two major differences. Firstly, original samples were analyzed for their calcium content immediately following their preparation, whereas those used as internal standards were stored in chemically inert vials for between 1 and 12 weeks. This group of analyses had to be delayed because the instrument used for the calcium analysis was not readily accessible. Secondly, the animals which provided the internal standard values were housed in different quarters from those used in the original study.

For the greater part, only the results obtained from the combined data are described in the following pages, but in instances where the two separate sets of data do not coincide, a detailed description of the discrepancies is presented. Some of these discrepancies are described in the text only; however, in cases which show differences that bear directly upon the specific conclusions to be drawn, the points of issue are shown

by means of overlays on the illustrations in addition to the [illegible] appearing in the text. Statistically significant differences [illegible] found between original and internal standard values are indi[illegible] overlays as 'p' values at the points involved.

A total of 55 means were obtained from the interna[illegible] tion of this study which could be compared with correspondin[illegible] the original study. When the comparisons were made, it was [illegible] of the 55 comparisons showed the original calcium value to be [illegible] larger than the internal standard value, and the remainin[illegible] showed the internal standard to be higher than the origina[illegible] these comparisons were analyzed statistically by a weighted [illegible] 't' test, it was found that 11 of the 55 were significantly [illegible] and only 2 were very highly significant (<0.001). Further[illegible] very highly significant differences, the internal standard [illegible] value in one and the lower value in the other. These findin[illegible] that the differences observed between the two sets of data are [illegible] variation which could be anticipated in a study involvin[illegible] ple sizes ranging from 3-6 measurements. The possible sour[illegible] tion were those that are of a biological nature, as well as [illegible] ing from the complex experimental procedure which was emp[illegible] plexity of the procedure was compounded by the necessity [illegible] the two sets of experiments under different conditions and [illegible] number of extremely old animals which were available throu[illegible] of the study.

Tissues Which Exhibit an Increased Calcium Content with A[illegible]

The calcium concentrations of the kidney at various [illegible]

by means of overlays on the illustrations in addition to the considerations appearing in the text. Statistically significant differences which were found between original and internal standard values are indicated on the overlays as 'p' values at the points involved.

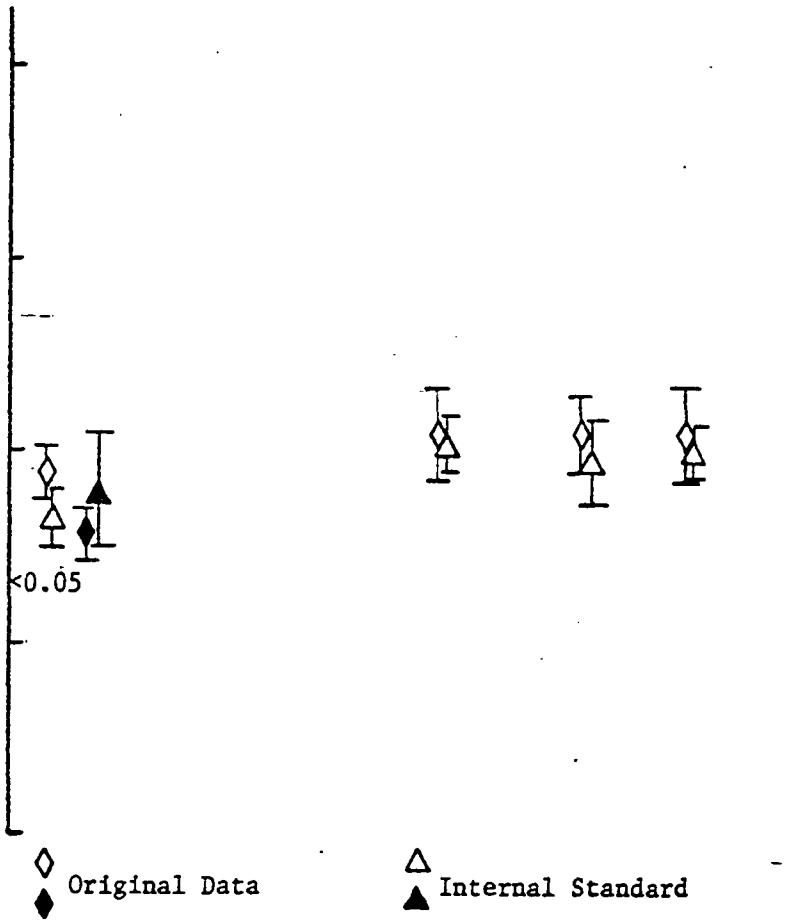
A total of 55 means were obtained from the internal standard portion of this study which could be compared with corresponding values from the original study. When the comparisons were made, it was found that 31 of the 55 comparisons showed the original calcium value to be numerically larger than the internal standard value, and the remaining 21 comparisons showed the internal standard to be higher than the original value. When these comparisons were analyzed statistically by a weighted Student's 't' test, it was found that 11 of the 55 were significantly different and only 2 were very highly significant (<0.001). Further, of the two very highly significant differences, the internal standard was the higher value in one and the lower value in the other. These findings suggest that the differences observed between the two sets of data are due to variation which could be anticipated in a study involving means of sample sizes ranging from 3-6 measurements. The possible sources of variation were those that are of a biological nature, as well as those resulting from the complex experimental procedure which was employed. The complexity of the procedure was compounded by the necessity of performing the two sets of experiments under different conditions and by the small number of extremely old animals which were available throughout the course of the study.

Tissues Which Exhibit an Increased Calcium Content with Aging

The calcium concentrations of the kidney at various ages are

shown in Figure 3. The results of the statistical analyses (Table 4) indicate that in the shorter-lived Fischer rats, the kidney calcium level does not increase late in life. However, at younger ages, viz., 2 and 4 months, the values obtained are significantly higher than the values on either side of 2 and 4 months and therefore may represent aberrant values. The kidneys of the longer-lived A x C strain demonstrate a gradual increase in the calcium content by age 16 months and a statistically significant increase in the oldest (27-30 months) group. The kidneys of the rats which live longer show the larger increase in calcium content which implies that kidney calcium content is a function of chronological age and involves a progressive mechanism for the accumulation of calcium. It must be realized, however, that the only group showing a significant increase in kidney calcium represents a small number of animals. However, the trend of a gradually increasing calcium content is upheld by this small group of A x C rats. Inspection of the internal standard data reveals some minor differences from those of the original data (see overlay of Figure 3). In all cases except the one-month-old Fischer rats, the general trends are not significantly altered.

The changes observed in lung calcium levels are presented in Figure 4. Statistical analyses (Table 5) indicate that the first significant increases in lung calcium contents occur at 19 and 27-30 months of age in the Fischer and A x C rats respectively. The significant drop in lung calcium content between the ages of 19 and 21 months in the Fischer rats represents a phenomenon that is unique among the tissues studied. While a satisfactory explanation cannot be given with certainty, the sharp reversal in direction seen in the calcium content of the Fischer



Kidney mean calcium values showing apparent discrepancies.

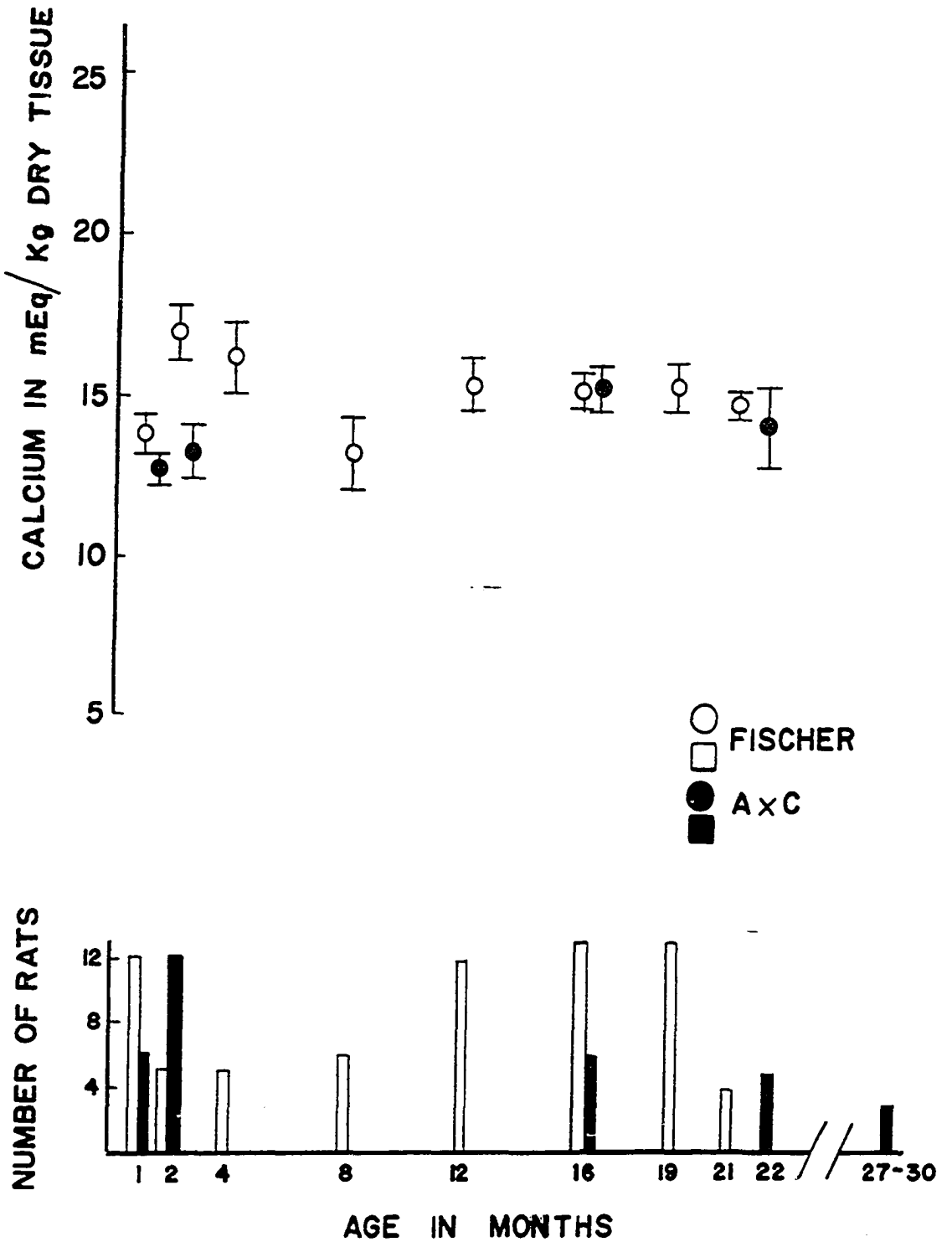


Figure 3. Kidney calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

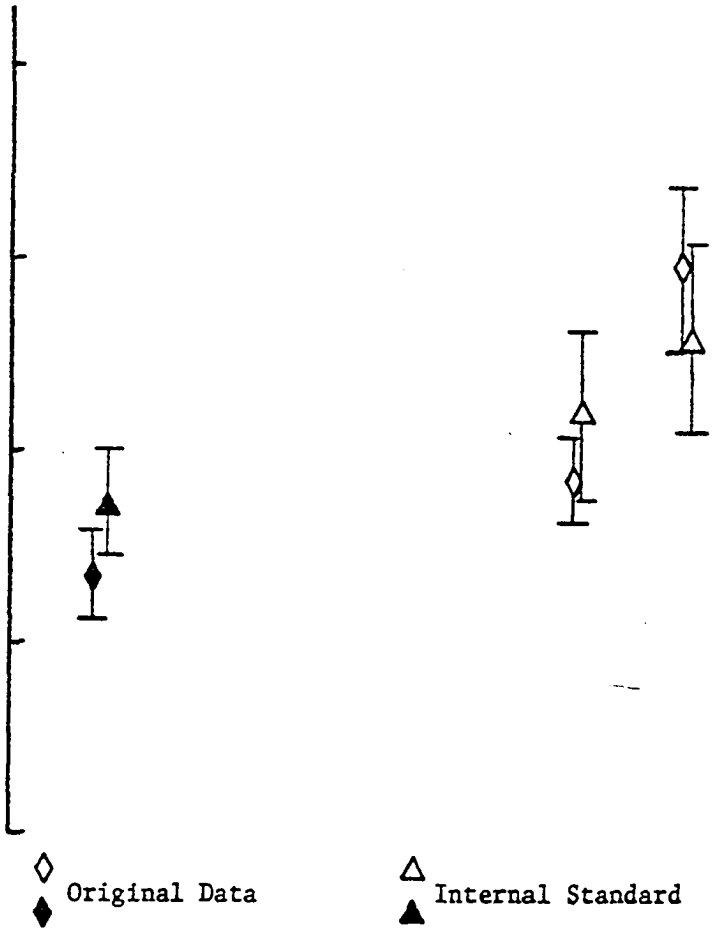
TABLE 4

NEW MULTIPLE RANGE TEST FOR KIDNEY CALCIUM CONTENT IN FISCHER AND A x G RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	13.86 ±.29	*	-	-	-	-	-	-		
5	2	.....	17.08 ±.81	-	*	-	-	-	-		
5	4	.....	.....	16.22 ±1.06	*	-	-	-	-		
6	8	.....	.....	.....	13.33 ±1.07	-	-	-	-		
12	12	.....	.....	.....	.....	15.13 ±.43	-	-	-		
13	16	.....	.....	.....	.....	.....	14.97 ±.43	-	-		
13	19	.....	.....	.....	.....	.....	.....	15.03 ±.44	-		
4	21	.....	.....	.....	.....	.....	.....	.....	14.80 ±.44		
					A x C						
6	1	12.92 ±.37	-				-			-	*
12	2	.....	13.39 ±.60				-			-	*
6	16	.....	.....	.....	.....	.....	15.35 ±.64			-	*
5	22	.....	.....	.....	.....	.....	.....	.....	.....	14.03 ±1.22	*
3	27-30	.....	.....	.....	.....	.....	.....	.....	.....	.....	18.49 ±.32

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.



Lung mean calcium values showing apparent discrepancies.

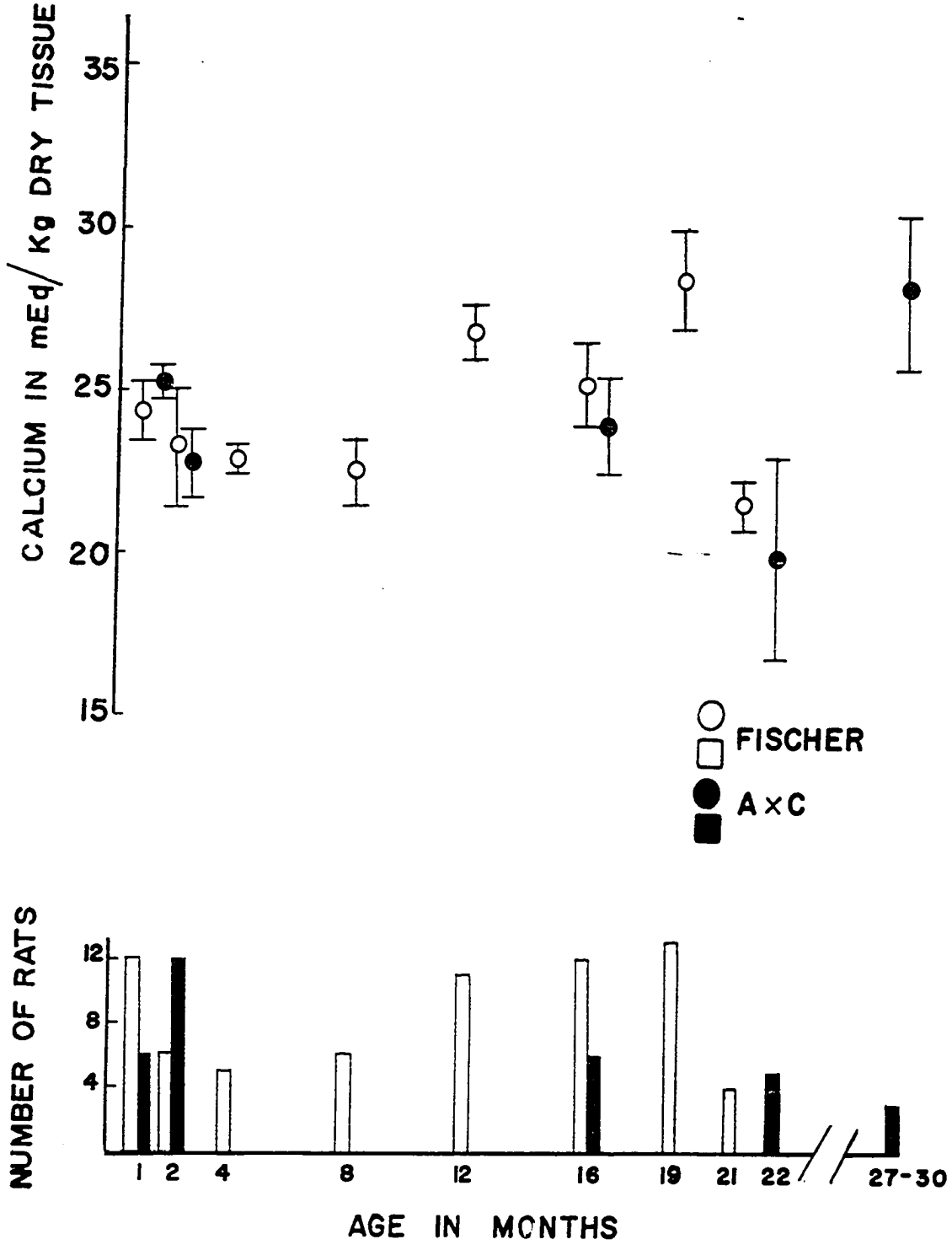


Figure 4. Lung calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 5

NEW MULTIPLE RANGE TEST FOR LUNG CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	24.33 ±.67	-	-	-	-	-	-	-		
6	2	.....	23.37 ±1.75	-	-	-	-	-	-		
5	4	.....	.....	23.01 ±.38	-	-	-	*	-		
6	8	.....	.....	.....	22.64 ±.90	-	-	*	-		
11	12	.....	.....	.....	.....	26.81 ±.52	-	-	*		
12	16	.....	.....	.....	.....	.....	25.07 ±1.00	-	-		
13	19	.....	.....	.....	.....	.....	.....	28.60 ±1.35	*		
4	21	.....	.....	.....	.....	.....	.....	.....	21.52 ±.71		
					A x C						
6	1	25.32 ±.04	-				-			-	-
12	2	.....	22.67 ±.68				-			-	-
6	16	.....	.....				24.05 ±1.56			-	-
5	22	.....	.....				.....			19.92 ±3.06	*
3	27-30	.....	.....				.....			.....	28.16 ±2.41

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

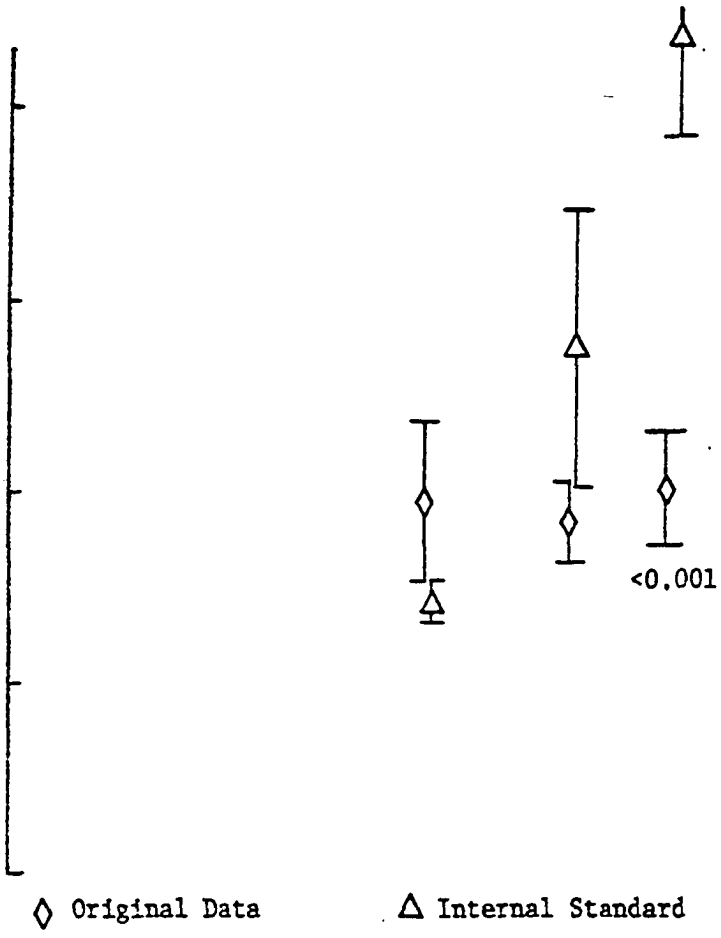
[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

lungs may be the result of the presence of a pathological lesion. No such lesion, however, was grossly visible in any of the tissue samples investigated. The author tends to place more credence in the higher calcium value (19 months) because of the comparatively larger number of animals included in that value than in the unexpectedly low 21 month value. Furthermore, the overlay of Figure 4 shows clearly that the upward trend of 19 months is present in both the original data and in the internal standard.

Unlike the kidney tissue, the lung accumulates calcium more rapidly in the shorter-lived Fischer strain. Such a rapid accumulation may be linked with a loss of functional capacity in the lungs of the Fischer rats which does not occur until later in the longer-lived A x C rats. The A x C rats attain a lung calcium concentration which is as high as that observed in the Fischer rats, but the increase does not occur until an older age is achieved, i.e., 27-30 months. Furthermore, this high value is significantly higher than the 22 month value only, one which appears to be lower than might have been anticipated from the other data points from this tissue. This finding suggests that some process which is accompanied by a relatively early and rapid increase in the calcium content of the lungs of Fischer rats may play a role in the inability of that strain to attain ages comparable to those reached by the A x C rats. Due to the erratic nature of the values obtained from lung tissue, a statement of definite age-related calcium changes is difficult to propose. Some fairly high calcium values do occur during the latter portion of life in both rat strains, and therefore the lung will be counted among those tissues which may accumulate calcium during aging.

Figure 5 shows the calcium changes which are observed during aging in the cerebral cortex. The results presented in Table 6 indicate that the significant increase in the calcium content of this region of the brain occurs in both rat strains, but to a greater degree in the shorter-lived Fischer rats. The calcium accumulation in the cerebral cortex is more rapid and quantitatively larger in the shorter-lived strain, whereas that seen in the longer-lived A x C rats is a more progressive occurrence. This finding indicates that the increasing calcium content of the cerebral cortex follows a pattern which could be associated with the shortness of life exhibited by the Fischer rats and could contribute to a loss of functional capacity. It can be seen from Figure 5 and its overlay that the sharp accumulation of calcium by the cerebral cortex occurs some two months later in the original data than in the corresponding internal standard values. This divergence is probably the result of simple biological variation since the combined data at 19 months is practically the same as the value obtained at an age of 21 months. Because of the larger number of animals included in the combined data, it is reasonable to conclude that the calcium buildup in the cerebral cortex generally occurs by the age of 19 months in the Fischer strain. This indication lends further support to the conclusion that the Fischer cerebral cortex is vulnerable to a large calcium accumulation at an earlier age than is that of the A x C.

The findings cited above indicate that in those tissues in which the calcium content rises during aging, the rise occurs during the second half of the life span for the most part, and the changes seen in the two strains are not comparable from the standpoint of either the age at the



Cerebral Cortex mean calcium values showing apparent discrepancies.

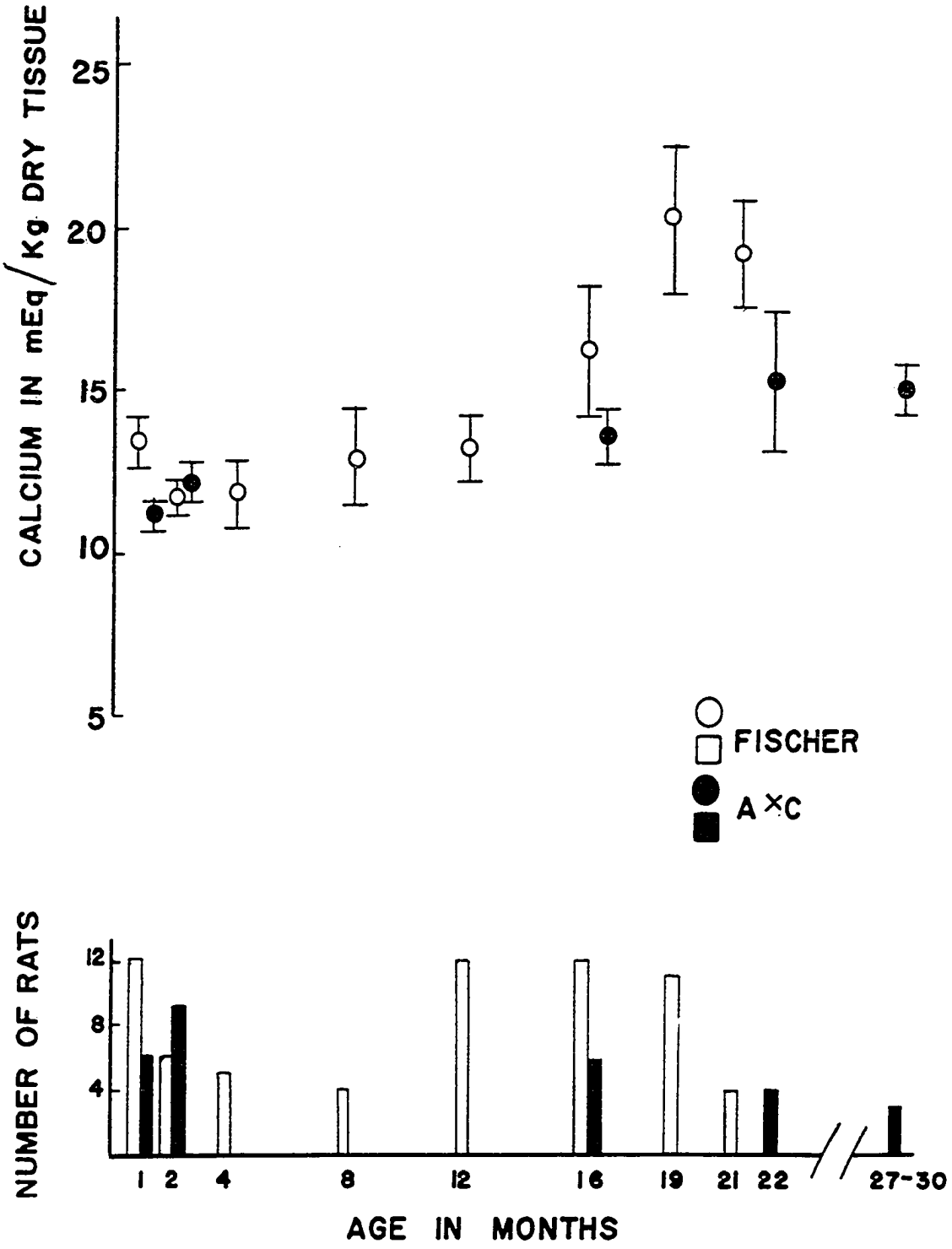


Figure 5. Cerebral cortex calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 6

NEW MULTIPLE RANGE TEST FOR CEREBRAL CORTEX CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	13.52 ±.49	-	-	-	-	-	-	-		
6	2		11.79 ±.40	-	-	-	-	*	*		
5	4			11.89 ±1.01	-	-	-	-	-		
4	8				13.03 ±1.51	-	-	-	-		
12	12					13.36 ±.87	-	-	-		
12	16						16.38 ±1.74	-	-		
11	19							20.37 ±2.17	-		
4	21								19.42 ±1.64		
					A x C						
6	1	11.39 ±.30	-				-			*	*
10	2		12.26 ±.25				-			-	-
6	16						13.62 ±.71			-	-
4	22									15.33 ±2.14	-
3	27-30										15.15 ±.79

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

time of onset or the magnitude of the change.

In the case of calcium accumulation by kidney tissue, the amount of increase in calcium content appears to be determined by how long the animal lives, since the few available longer-lived A x C rats exhibited a greater increase in kidney calcium content than did the Fischer rats. Conversely, in the cerebral cortex, and possibly in the lung, the amount and rate of calcium accumulation appear to be factors which may be related to the length of the life span which can be achieved.

Tissues Which Exhibit Calcium Loss with Aging

The tissues which show significant losses of calcium during aging, i.e., skin, aorta, cardiac and skeletal muscle (Figures 6-9), do so during the first half of life in both strains. After middle-age is attained, none of these tissues exhibit any major change in calcium content (Tables 7-10). This finding indicates that the loss of calcium observed is more closely related to growth and development than to the processes of senescence which affect calcium accumulation.

In the skin of both rat strains (Figure 6), no statistically significant changes in calcium content are observed after the age of 16 months and the calcium content is maintained at that level for the remainder of the life span. When the original data were compared with the internal standard values, a statistical difference occurred in the A x C rats at the age of two months, but the pattern exhibited by both sets of data was still that of a decreasing calcium concentration, as shown by the combined data presented here.

The aortic calcium content declines during the first half of the life-span in both rat strains (Figure 7), and the level attained at the

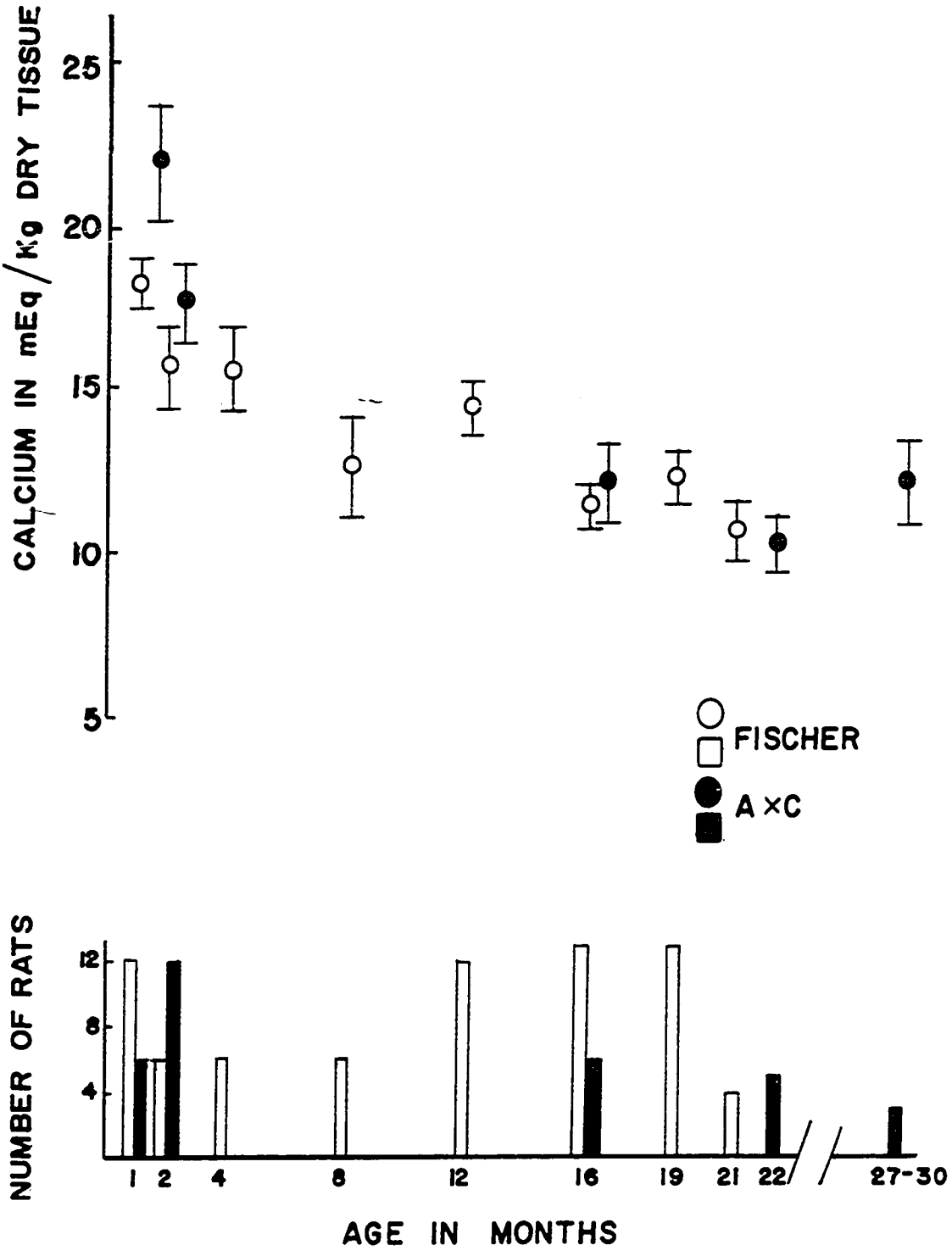


Figure 6. Skin calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 7

NEW MULTIPLE RANGE TEST FOR SKIN CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	18.19 ±.50	-	-	*	-	*	*	*		
6	2	.....	15.81 ±1.37	-	-	-	*	-	*		
6	4	.....	.....	15.66 ±1.22	-	-	*	-	*		
6	8	.....	.....	.....	12.70 ±1.60	-	-	-	-		
12	12	.....	.....	.....	.....	14.57 ±.61	-	-	*		
13	16	.....	.....	.....	.....	.....	11.46 ±.49	-	-		
13	19	.....	.....	.....	.....	.....	.....	12.47 ±.58	-		
4	21	.....	.....	.....	.....	.....	.....	.....	10.88 ±.64		
					A x C						
6	1	22.06 ±1.71	-				*			*	*
12	2	.....	17.83 ±.82				-			*	*
6	16	.....	.....	.....	.....	.....	12.32 ±1.11			-	-
5	22	.....	.....	.....	.....	.....	.....	.....	.....	10.25 ±.53	-
3	27-30	.....	.....	.....	.....	.....	.....	.....	.....	.....	12.21 ±1.32

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

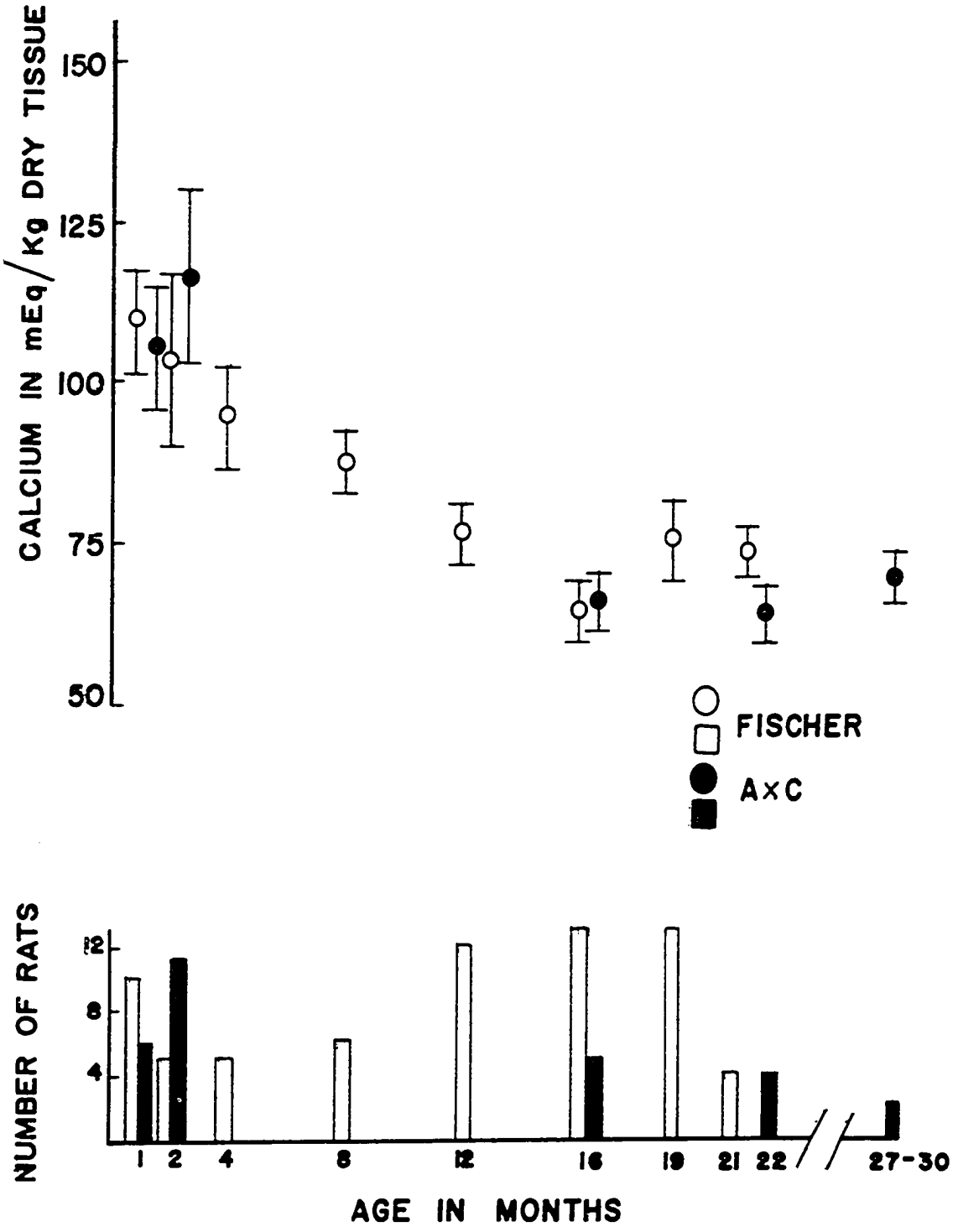


Figure 7. Aorta calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 8

NEW MULTIPLE RANGE TEST FOR AORTA CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
10	1	108.07 ±6.52	-	-	-	*	*	*	*		
5	2	.....	102.79 ±12.76	-	-	-	*	-	-		
5	4	.....	.....	95.03 ±7.98	-	-	*	-	-		
6	8	.....	.....	.....	88.17 ±10.72	-	*	-	-		
12	12	.....	.....	.....	.....	76.34 ±3.34	-	-	-		
13	16	.....	.....	.....	.....	.....	64.80 ±3.98	-	-		
13	19	.....	.....	.....	.....	.....	.....	75.56 ±5.24	-		
3	21	.....	.....	.....	.....	.....	.....	.....	74.51 ±3.90		
					A x C						
6	1	105.25 ±9.52	-				-			-	-
11	2	.....	117.58 ±12.32				-			*	-
5	16	.....	.....	.....	.....	.....	66.69 ±3.73			-	-
4	22	.....	.....	.....	.....	.....	.....	.....	.....	63.75 ±4.67	-
2	27-30	.....	.....	.....	.....	.....	.....	.....	.....	.....	69.57 ±3.68

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

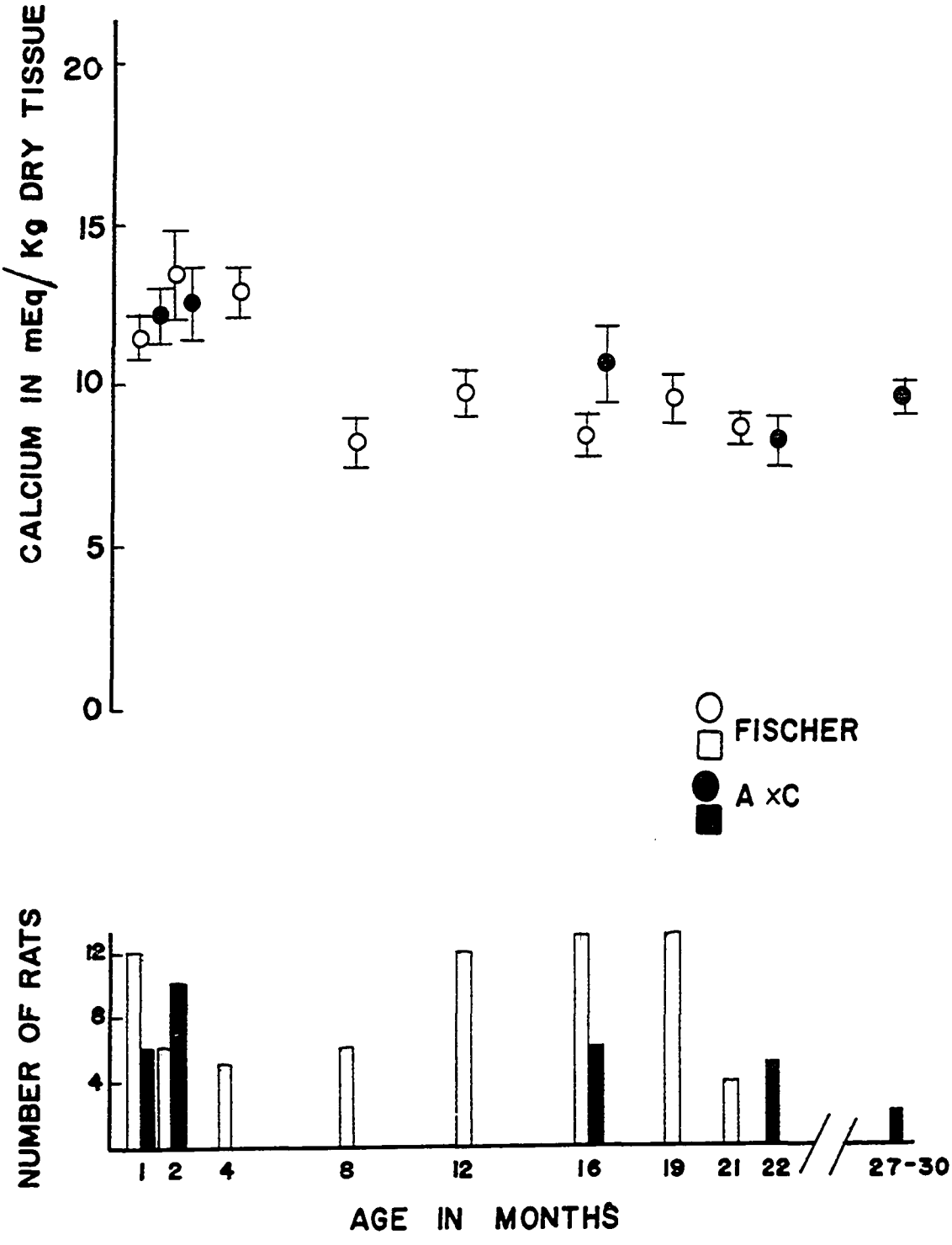


Figure 8. Cardiac muscle calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 9

NEW MULTIPLE RANGE TEST FOR CARDIAC MUSCLE CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	11.59 ±.30	-	-	-	-	-	-	-		
6	2	.....	13.49 ±1.43	-	*	*	*	*	*		
5	4	.....	.....	12.96 ±1.60	*	*	*	*	*		
6	8	.....	.....	.....	8.35 ±.63	-	-	-	-		
12	12	.....	.....	.....	.....	9.90 ±.35	-	-	-		
13	16	.....	.....	.....	.....	.....	8.52 ±.38	-	-		
13	19	.....	.....	.....	.....	.....	.....	9.59 ±.40	-		
3	21	.....	.....	.....	.....	.....	.....	.....	8.76 ±.32		
					A x C						
6	1	12.39 ±.76	-				-			-	-
11	2	.....	12.43 ±.98				-			-	-
6	16	.....	.....				10.66 ±1.20			-	-
5	22	.....	.....				.....			8.31 ±.53	-
2	27-30	.....	.....				.....			.....	9.65 ±.18

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

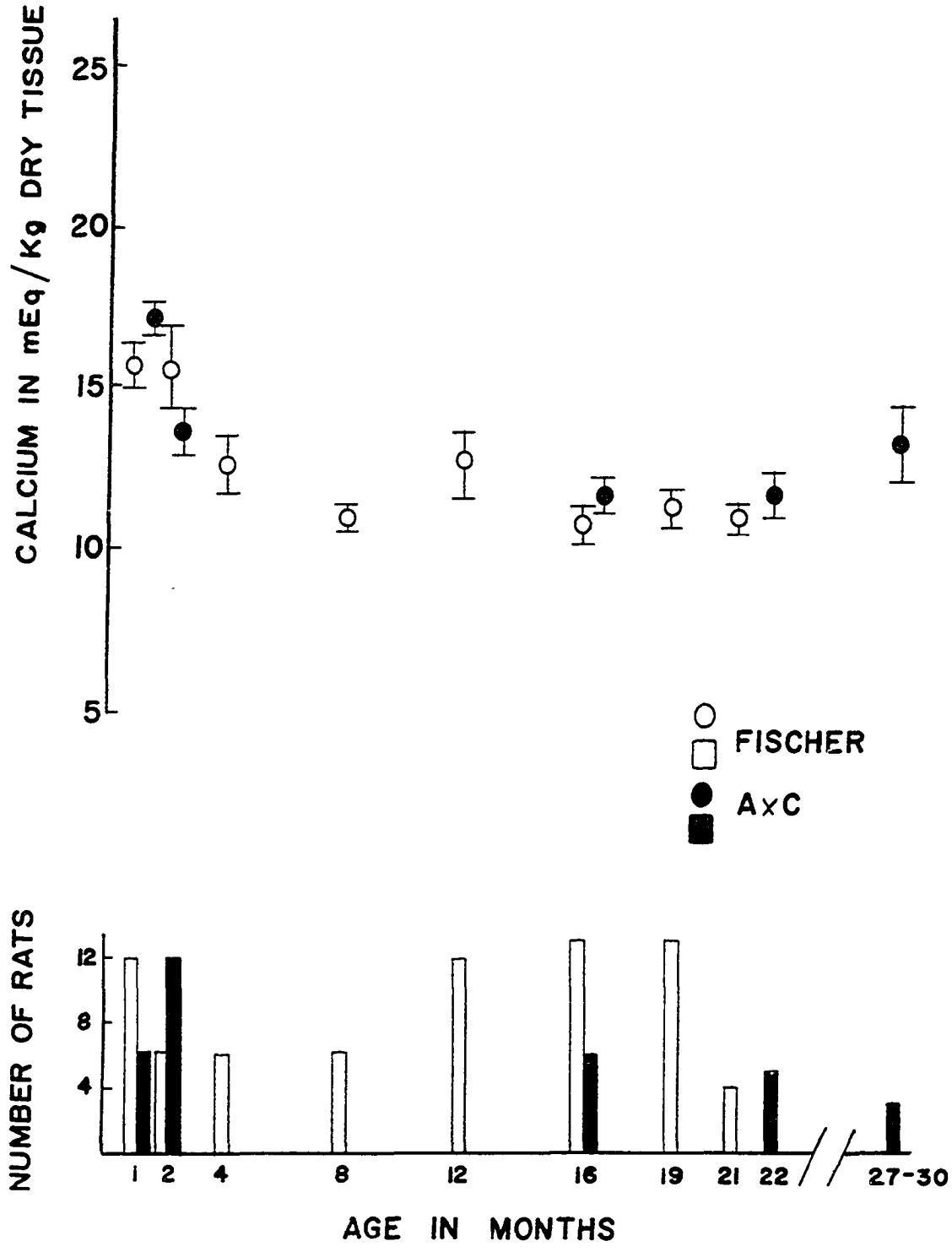


Figure 9. Skeletal muscle calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 10

NEW MULTIPLE RANGE TEST FOR SKELETAL MUSCLE CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	15.70 ±.54	-	-	*	-	*	*	*		
6	2	.....	15.75 ±1.33	*	*	*	*	*	*		
6	4	.....	.....	12.98 ±.69	-	-	-	-	-		
6	8	.....	.....	.....	11.17 ±.23	-	-	-	-		
12	12	.....	.....	.....	.....	12.85 ±.88	-	-	-		
13	16	.....	.....	.....	.....	.....	10.94 ±.30	-	-		
13	19	.....	.....	.....	.....	.....	.....	11.46 ±.28	-		
4	21	.....	.....	.....	.....	.....	.....	.....	11.14 ±.10		
					A x C						
6	1	17.27 ±.41	*				*			*	*
12	2	.....	13.84 ±.45				-			-	-
6	16	.....	.....				11.91 ±.44			-	-
5	22	.....	.....	.....	.....	.....	.....	.....	.....	11.65 ±.29	-
3	27-30	.....	.....	.....	.....	.....	.....	.....	.....	.....	13.35 ±1.12

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

ages of 12 and 16 months in the Fischer and A x C rats respectively remains unchanged during the balance of the life-spans (Table 8). Although a statistically significant difference was observed between the original and internal standard values in the Fischer aortic tissue at 16 months of age, the combined data are not markedly affected by this discrepancy and the age-related calcium pattern in both sets of data is clearly a downward slope. The large variability which occurs in the measurement of calcium in aortic tissue is accompanied by numerically large mean calcium values which are from 6-15 times larger than those obtained from analyses of the other tissues investigated.

Both cardiac and skeletal muscle tissues show a decline in calcium content at early ages in the two rat strains (Figures 8 and 9) with no significant changes occurring during the last half of life (Tables 9 and 10). In both of these tissues, the original and internal standard values were virtually identical.

The changes observed in calcium content of the tissues described in this section are probably the result of changes in the proportions of the various tissue constituents with which calcium is associated, e.g., fluid compartments and connective tissue.

This study has shown that in those tissues which lose calcium during the life-span, the rates of loss appear to be dissimilar in the two rat strains, but the amounts of calcium present after middle age is reached are essentially identical in both strains. These findings imply that the rate of development at the tissue level differs in these rats having dissimilar life spans, but that by the time middle-age is reached, the two strains are very similar from a developmental standpoint.

Tissues with Constant Calcium Levels Throughout Life

The calcium concentrations of liver, intestinal smooth muscle, plasma and whole blood at various ages are shown in Figures 10, 11, 12 and 13 respectively. The results of the statistical analyses of these data (Tables 11-14) show that no statistically significant changes occur which seem to be related to aging.

The highest and lowest mean calcium values obtained on the liver tissue of A x C rats are significantly different (Table 11). However, the difference does not appear to have any direct relationship to aging processes.

Intestinal smooth muscle shows a considerable degree of variability (Figure 11), and no statistically significant differences are demonstrable (Table 12). Although the calcium content of this tissue appears quite variable, none of the variations observed in this study can be linked to phenomena associated with aging.

The calcium concentration of both plasma (Figure 12) and whole blood (Figure 13) show no statistically significant changes throughout the life-span of either rat strain investigated (Tables 13 and 14). The mean calcium values obtained from both plasma and whole blood were found to lie within a range of one milliequivalent per liter throughout the entire life-span of the two rat strains. This finding is another demonstration of the remarkable constancy of the calcium levels in blood components during the lives of these animals. Figures 12 and 13 are each accompanied by overlays which show that, in general, the internal standard values are significantly lower than the original ones. Although the differences observed have little bearing on the age-related patterns

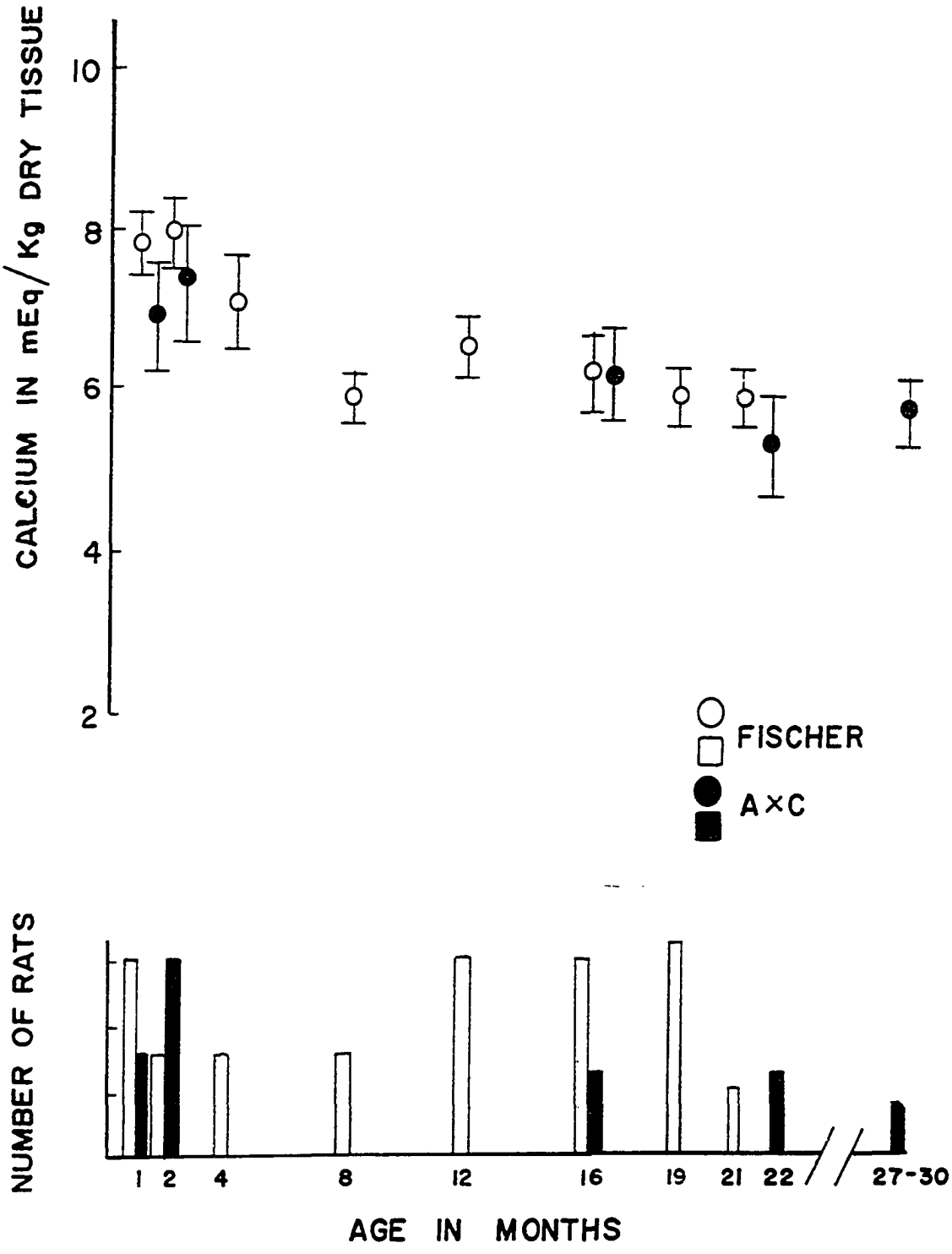


Figure 10. Liver calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 11

NEW MULTIPLE RANGE TEST FOR LIVER CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	7.82 ±.32	-	-	-	-	-	-	-		
6	2	.....	7.96 ±.55	-	-	-	-	-	-		
6	4	.....	.....	7.11 ±.62	-	-	-	-	-		
6	8	.....	.....	.....	5.86 ±.36	-	-	-	-		
11	12	.....	.....	.....	.....	6.54 ±.26	-	-	-		
12	16	.....	.....	.....	.....	.....	6.19 ±.41	-	-		
13	19	.....	.....	.....	.....	.....	.....	5.88 ±.21	-		
6	21	.....	.....	.....	.....	.....	.....	.....	5.82 ±.36		
					A x C						
6	1	6.99 ±.62	-				-			-	-
12	2	.....	7.37 ±.29				-			*	-
5	16	.....	.....				6.13 ±.58			-	-
5	22	.....	.....				.....			5.32 ±.53	-
3	27-30	.....	.....				.....			.....	5.71 ±.40

[a]Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

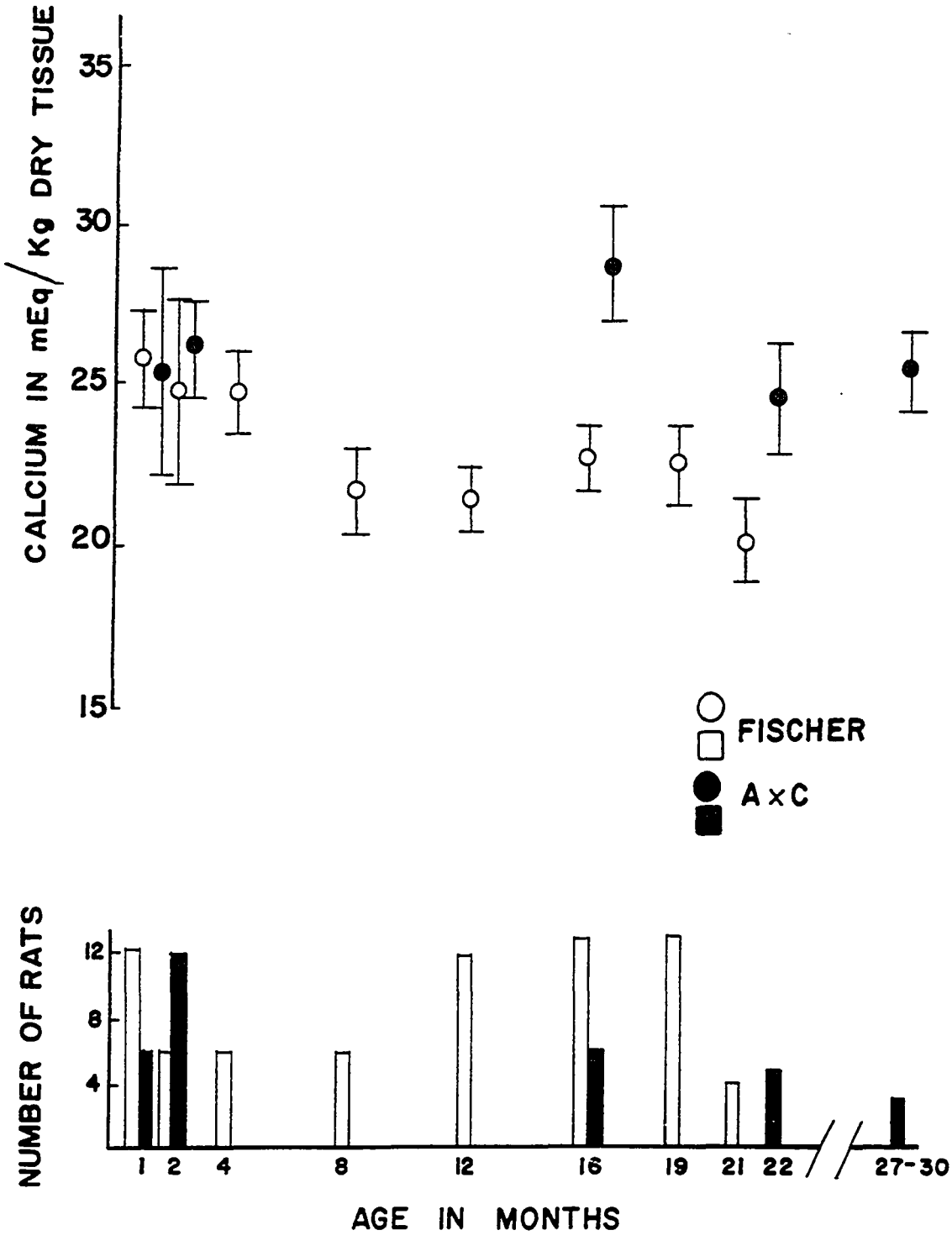


Figure 11. Intestinal smooth muscle calcium concentration as a function of age. Top: Calcium concentrations in mEq/kg dry tissue in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

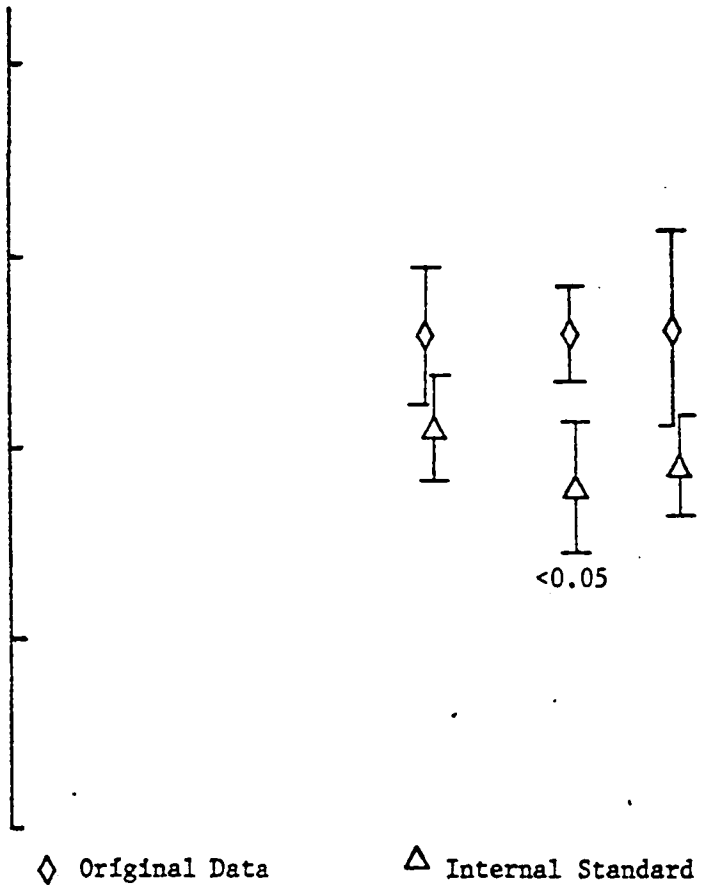
TABLE 12

NEW MULTIPLE RANGE TEST FOR INTESTINAL SMOOTH MUSCLE CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	25.60 ±1.23	-	-	-	-	-	-	-		
6	2	.....	24.68 ±2.96	-	-	-	-	-	-		
6	4	.....	.....	24.68 ±3.10	-	-	-	-	-		
6	8	.....	.....	.....	21.68 ±1.60	-	-	-	-		
12	12	.....	.....	.....	.....	21.38 ±.71	-	-	-		
13	16	.....	.....	.....	.....	.....	22.64 ±.61	-	-		
13	19	.....	.....	.....	.....	.....	.....	22.48 ±1.01	-		
4	21	.....	.....	.....	.....	.....	.....	.....	20.13 ±1.22		
					A x C						
6	1	25.28 ±3.25	-				-			-	-
12	2	.....	25.99 ±1.44				-			-	-
6	16	.....	.....				28.76 ±1.55			-	-
5	22	.....	.....				.....			24.44 ±1.70	-
3	27-30	.....	.....				.....			.....	25.22 ±1.28

[a] Values shown are mean calcium ± S.E. in mEq/kg dry weight.

[b] Symbols: * = significant at the 0.01 level; (-) = not significant.



Plasma mean calcium values showing apparent discrepancies

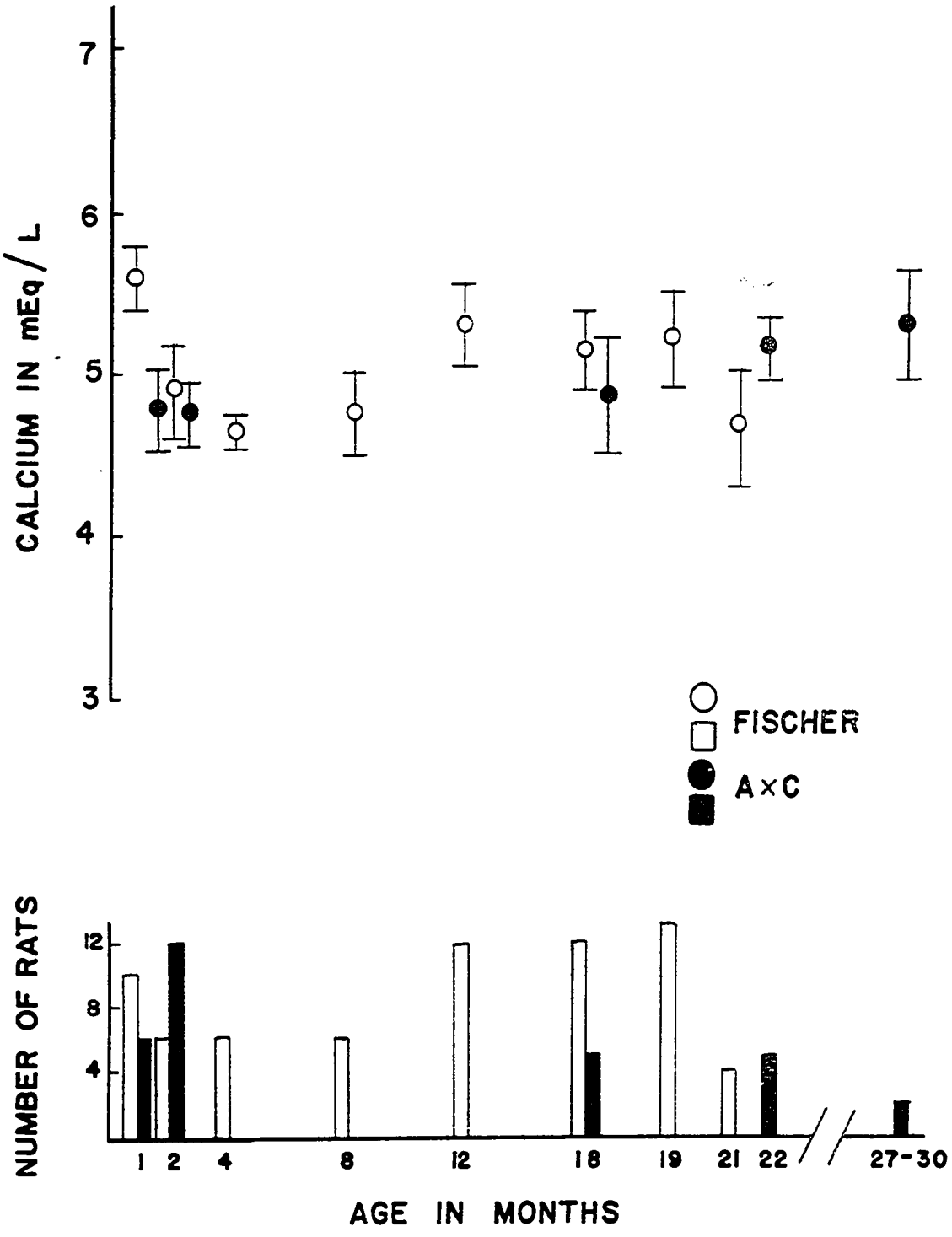


Figure 12. Plasma calcium concentration as a function of age. Top: Calcium concentrations in mEq/l of plasma in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

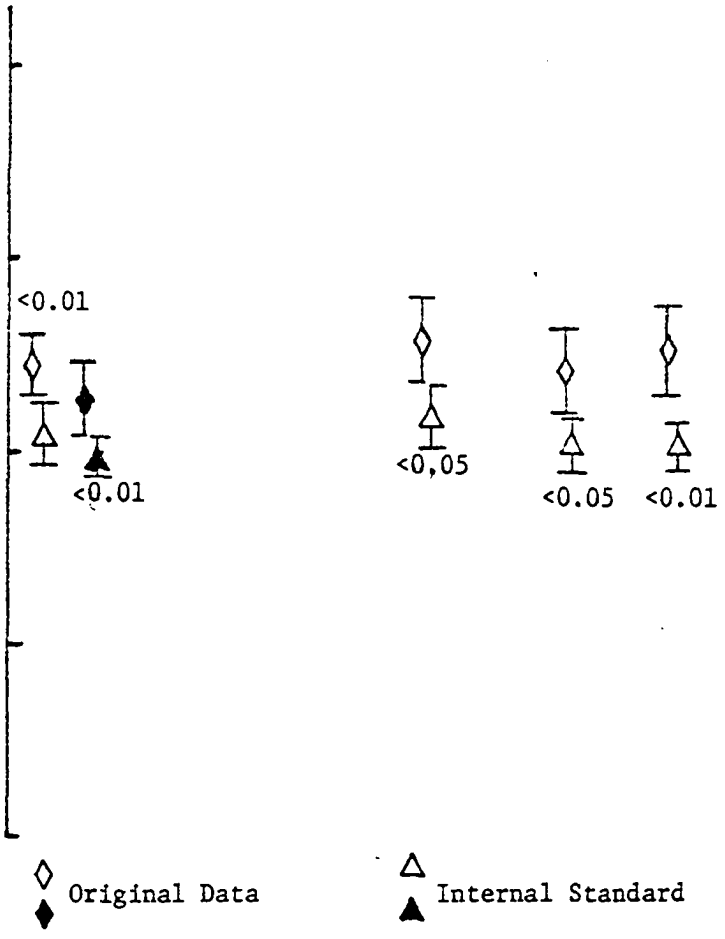
TABLE 13

NEW MULTIPLE RANGE TEST FOR PLASMA CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
10	1	5.63 ±.13	-	-	-	-	-	-	-		
6	2	.....	4.90 ±.29	-	-	-	-	-	-		
6	4	.....	.....	4.69 ±.09	-	-	-	-	-		
6	8	.....	.....	.....	4.74 ±.25	-	-	-	-		
12	12	.....	.....	.....	.....	5.32 ±.20	-	-	-		
12	16	.....	.....	.....	.....	.....	5.14 ±.20	-	-		
13	19	.....	.....	.....	.....	.....	.....	5.19 ±.25	-		
4	21	.....	.....	.....	.....	.....	.....	.....	4.68 ±.38		
					A x C						
6	1	4.81 ±.23	-				-			-	-
12	2	.....	4.75 ±.16				-			-	-
5	16	.....	.....				4.87 ±.39			-	-
5	22	.....	.....				.....			5.14 ±.12	-
2	27-30	.....	.....				.....			.....	5.28 ±.32

[a]Values shown are mean calcium ± S.E. in mEq/liter.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.



Whole Blood mean calcium values showing apparent discrepancies.

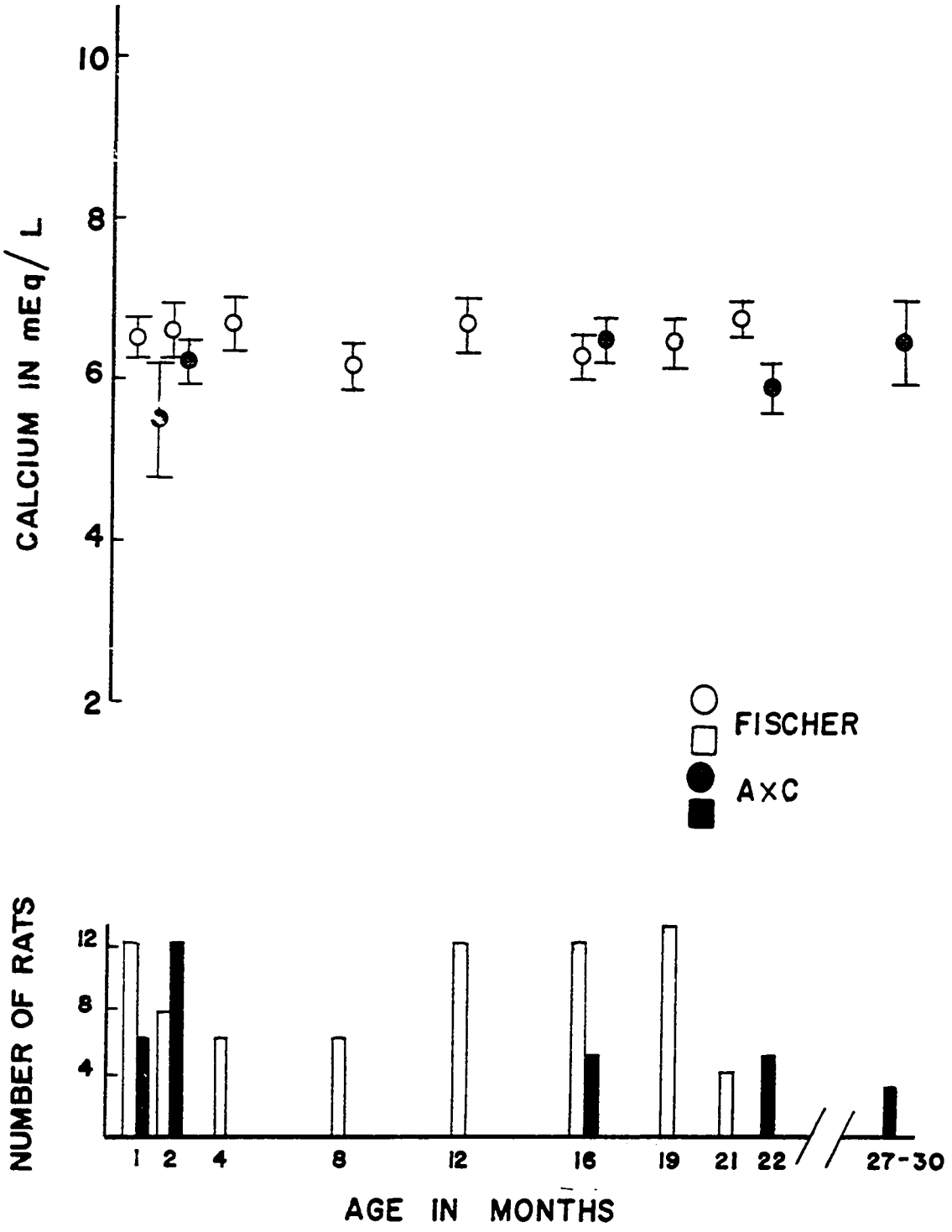


Figure 13. Whole blood calcium concentration as a function of age. Top: Calcium concentrations in mEq/l of whole blood in Fischer (○) and A x C (●) rats. I-bars represent standard errors of mean values. Bottom: Number of Fischer (□) and A x C (■) rats represented by corresponding mean calcium values.

TABLE 14

NEW MULTIPLE RANGE TEST FOR WHOLE BLOOD CALCIUM CONTENT IN FISCHER AND A x C RATS[a,b]

n	Age in Months	1	2	4	8	12	16	19	21	22	27-30
					Fischer						
12	1	3.25 ±.08	-	-	-	-	-	-	-		
6	2	.....	3.28 ±.15	-	-	-	-	-	-		
6	4	.....	.....	3.31 ±.17	-	-	-	-	-		
6	8	.....	.....	.....	3.08 ±.09	-	-	-	-		
12	12	.....	.....	.....	.....	3.34 ±.10	-	-	-		
12	16	.....	.....	.....	.....	.....	3.17 ±.09	-	-		
13	19	.....	.....	.....	.....	.....	.....	3.21 ±.10	-		
4	21	.....	.....	.....	.....	.....	.....	.....	3.35 ±.03		
					A x C						
6	1	2.75 ±.35	-				-			-	-
12	2	.....	3.10 ±.07				-			-	-
5	16	.....	.....				3.24 ±.10			-	-
5	22	.....	.....	.....	.....	.....	.....	.....	.....	2.94 ±.08	-
3	27-30	.....	.....	.....	.....	.....	.....	.....	.....	.....	3.20 ±.23

[a]Values shown are mean calcium ± S.E. in mEq/liter.

[b]Symbols: * = significant at the 0.01 level; (-) = not significant.

observed, the consistently lower numerical values for the internal standards require further evaluation. It should be noted that the numerical differences are quite small in absolute terms, and that the mean calcium concentrations in these samples are also relatively low. A number of possible factors could contribute to the discrepancies noted. Firstly, the two sets of samples were handled in slightly different ways, e.g.,subjected to a longer period of storage prior to analysis. Secondly, the standard calcium solutions used may have differed slightly between the two sets of data. A very slight difference in the standards might be evident in the analysis of these low calcium containing samples but might not be apparent in other samples containing larger amounts of calcium and having greater variability from other sources. Thirdly, the animals in the internal standard portion of this investigation might have had plasma and whole blood calcium concentrations which were actually lower than those observed in the original study. The latter possibility can probably be ruled out by the findings reported earlier which showed that approximately one-half of the internal standard values were higher and one-half lower than the original values. Of the first two possibilities described, the second is the more attractive, because low calcium concentrations and the low variability, especially in whole blood samples, would allow a slight difference in the calcium standard solution to exert a noticeable effect. Such an effect would be much less evident in the tissues in which absolute values and variations were larger. It is important, however, to reiterate that when the two sets of data are combined, no age-related changes in the calcium concentrations of either plasma or whole blood are present.

Summary of the Findings Within the Strains

The qualitative aspects of the age-related findings of the tissue calcium contents investigated are presented below:

(1) The kidneys, lungs and cerebral cortex tend to accumulate calcium during aging. When such an accumulation occurs, it is accomplished late in life in both rat strains investigated.

(2) The skin, aorta, cardiac and skeletal muscle contain a lower calcium concentration at middle-age than at one month of age, but little change occurs after middle-age is attained.

(3) The calcium concentrations of liver, intestinal smooth muscle, plasma and whole blood remain essentially unchanged throughout life in both rat strains.

Findings Between Strains

Relationship of Chronological Age to Calcium Changes in the Two Rat Strains

This section considers the dissimilarities observed between the strains in relation to calcium alterations. In those tissues which exhibit either an increase or decrease in calcium content during life, the alterations are usually achieved at different ages in the two rat strains (right hand columns of Table 15). The largest percentage of difference in calcium content achieved in each tissue is shown in the central columns of Table 15, even though some of the differences are not statistically significant.

Table 15 shows that in three of the four tissues which exhibit a loss of calcium during aging, i.e., skin, aorta, cardiac and skeletal muscle, the longer-lived A x C rats show a greater percentage of loss than do

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TABLE 15

TOTAL PERCENTAGE OF DIFFERENCE IN TISSUE CALCIUM CONTENT AND CHRONOLOGICAL AGE WHEN THE CHANGE IS ACHIEVED IN FISCHER AND A x C RATS

Type of Change	Tissues	Total Percentage of Net Difference During Life		Age in Months When Change Is Achieved	
		Fischer	A x C	Fischer	A x C
Accumulation Late in Life					
	Kidney	22	30	--	27-30
	Lung	25	29	21	27-30
	Cerebral cortex	42	26	19	22
Loss Early in Life					
	Skin	40	54	21	22
	Aorta	40	46	16	22
	Cardiac muscle	38	33	8	--
	Skeletal muscle	31	33	16	22
No Apparent Age-related Change					
	Liver	27	28	--	22
	Intestinal smooth muscle	22	15	--	--
	Plasma	17	10	--	--
	Whole blood	8	15	--	--

[a](--) = no significant difference throughout the life span.

the Fischer rats. Such a pattern is not evident in the tissues which tend to accumulate calcium, i.e., kidney, lung and cerebral cortex. This finding implies that in the longer-lived rats more calcium is lost from those tissues in which a decline is observed. On the other hand, the accumulation of calcium by tissues seems to be accomplished by a mechanism which is not directly determined by the length of life of the animal.

The largest significant change in tissue calcium content is consistently achieved at earlier chronological ages in the Fischer strain in each tissue investigated (right hand columns of Table 15). In general, the major strain difference in the age-related tissue calcium alterations is probably the age at the time of the change. In addition, in some instances a difference also exists in the magnitude of the change.

Comparisons of the Tissue Calcium Contents in the Two Rat Strains at Equivalent Physiological Ages

Comparison of the calcium contents of the various tissues in the two rat strains at ages which represent equivalent portions of the respective life spans would show whether differences exist at periods of life which are physiologically comparable. The physiological ages referred to below are described in detail in Materials and Methods (page 35), and the ages selected for comparison represent the youngest, middle-aged and oldest animals of each strain of rats.

As was the case in the longitudinal findings reported earlier in this study, the most meaningful results of the comparisons made between strains are those from tissues which accumulate calcium during aging.

Table 16 shows that the kidney tissue of young A x C rats and of

TABLE 16

COMPARISON OF KIDNEY CALCIUM CONTENTS IN FISCHER AND A X C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A X C Ca (in mEq/kg dry tissue)[b]	Fischer Ca -A X C Ca[c]	Standard Error of the Difference	p value[d]
<5	12	13.86 ±.29	6	12.92 ±.37	0.94	0.48	n.s.
50-55	12	15.13 ±.43	6	15.35 ±.64	-0.22	0.76	n.s.
>90	4	14.80 ±.44	3	18.49 ±.44	-3.69	0.59	<0.01

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A X C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A X C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II, (page 24).

young Fischer rats have similar calcium contents. Yet the kidneys of oldest A x C animals have a calcium content significantly above that of the oldest Fischer rats. These findings, as well as the longitudinal ones, suggest that the longer life span of the A x C animals predisposes kidney tissue to greater calcification. It should be noted, however, that results from these older groups are less reliable because they contained a smaller number of animals.

In the lung (Table 17) no difference is seen at any of the three ages compared, while in the cerebral cortex (Table 18) the two strains have different calcium contents at equivalent young ages. These differences disappear as the animals reach maturity, and the calcium values remain similar in the oldest age groups compared. Therefore, the calcium levels in these tissues appear to be related to the life span of the animals, rather than to the chronological ages. That is, when the calcium contents of the lung and cerebral cortex are viewed in each strain separately, the shorter-lived Fischer rats accumulate calcium more rapidly and to a higher concentration than the A x C animals. Yet, when rats of both strains have reached the final stages of their respective life spans, the calcium levels are no longer significantly different.

None of the tissues exhibiting a calcium loss during life (Tables 19-22) show a significant difference between the two strains at equivalent physiological ages with the exception of the skin in the youngest animals. This general absence of strain differences among tissues which tend to lose calcium during aging suggests that the calcium content of these tissues is dependent on the stage of development of the animal, rather than the chronological age. The different calcium pictures seen

TABLE 17

COMPARISON OF LUNG CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C Ca (in mEq/kg dry tissue)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
< 5	12	24.32 ±.67	6	25.32 ±.04	-1.00	0.96	n.s.
50-55	11	26.81 ±.52	6	24.05 ±1.56	2.76	1.33	n.s.
>90	4	21.52 ± .71	3	28.16 ±2.41	-6.64	3.44	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 18

COMPARISON OF CEREBRAL CORTEX CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C ca (in mEq/kg dry tissue)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
< 5	12	13.52 ± .49	6	11.39 ± .30	2.13	0.73	< 0.05
50-55	12	13.36 ± .87	6	13.62 ± .71	26	1.34	n.s.
>90	4	19.42 ±1.64	3	15.15 ± .79	4.27	2.05	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 19

COMPARISON OF SKIN CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C Ca (in mEq/kg dry tissue)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
<5	12	18.19 ±.50	6	22.06 ±1.71	-3.87	1.37	< 0.05
50-55	12	14.5 ±.61	6	12.32 ±1.11	2.25	1.16	n.s.
>90	4	10.88 ±.64	3	12.21 ±1.32	-1.33	1.34	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 20

COMPARISON OF AORTA CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C Ca (in mEq/kg dry tissue)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
< 5	10	108.07 ± 6.52	6	105.25 ± 9.52	-2.82	11.22	n.s.
50-55	12	76.34 ± 3.34	5	66.69 ± 3.73	9.65	5.72	n.s.
> 90	3	74.51 3.90	2	69.57 3.68	4.94	4.59	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 21

COMPARISON OF CARDIAC MUSCLE CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C Ca (in mEq/kg dry tissue)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
<5	12	11.59 ± .30	6	12.39 ± .76	-0.80	0.67	n.s.
50-55	12	9.90 ± .35	6	10.66 ± 1.20	-0.76	0.96	n.s.
>90	3	8.76 ± .32	2	9.65 ± .18	-0.89	0.43	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 22

COMPARISON OF SKELETAL MUSCLE CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C Ca (in mEq/kg dry tissue)	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
<5	12	15.70 ±.54	6	17.27 ±.41	-1.57	0.82	n.s.
50-55	12	12.85 ±.88	6	11.91 ±.44	0.94	1.30	n.s.
>90	4	11.14 ±.10	3	13.35 ±1.12	2.21	0.95	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

in the chronological comparisons of these tissues virtually disappear when the animals are compared at ages representing equivalent portions of the life spans.

Among those tissues in which the calcium concentration remains constant throughout life, some strain differences were observed. Although liver calcium content does not differ between the two strains (Table 23), intestinal smooth muscle has a higher calcium content in the A x C than in the Fischer rats during middle and old ages (Table 24). The plasma calcium concentration is higher in young Fischer than in young A x C rats (Table 25), but no significant differences occur between whole blood calcium concentrations in the two strains (Table 26). Because no calcium changes were seen in these tissues with aging, the observed differences in calcium levels in the two strains must result from other factors.

A summary of the findings just discussed is presented in Table 27. Of the 33 comparisons which were performed between the different rat strains (i.e., at the youngest, middle, and oldest ages in each of the eleven tissues), only 6, or less than 20 per cent, of the cases tested show statistically significant differences at equivalent physiological ages. For example, the kidney calcium content shows a significant strain difference in only one of the three comparisons made; liver shows no significant difference in any of the three comparisons. Of the 6 differences which are significant, 3 of them occur between animals of the youngest age groups which were compared, i.e., at ages representing <5 per cent of the total life spans. It should be emphasized that half of the strain differences in tissue calcium content occur early in life during the rapid growth stages. In addition, only 3 of the 6 instances where

TABLE 23

COMPARISON OF LIVER CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C Ca (in mEq/kg dry tissue)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
<5	12	7.82 ±.32	6	6.99 ±.62	0.83	0.62	n.s.
50-55	11	6.54 ±.26	5	6.13 ±.58	0.41	0.54	n.s.
>90	4	5.82 ±.36	3	5.71 ±.40	0.11	1.27	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 24

COMPARISON OF INTESTINAL SMOOTH MUSCLE CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/kg dry tissue)[b]	n	A x C Ca (in mEq/kg dry tissue)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
<5	12	25.60 ±1.23	6	25.28 ±3.25	0.32	2.84	n.s.
50-55	12	21.38 ± .71	6	28.76 ±1.55	-7.38	1.47	<0.01
>90	4	20.12 ±1.22	3	25.22 ±1.28	-5.10	1.86	<0.05

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 25

COMPARISON OF PLASMA CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/l)[b]	n	A x C Ca (in mEq/l)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
<5	12	5.63 ± .13	6	4.81 ± .23	0.82	0.24	<0.01
50-55	12	5.32 ± .20	5	4.87 ± .39	0.45	0.39	n.s.
>90	4	4.68 ± .38	2	5.28 ± .32	-0.60	0.60	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 26

COMPARISON OF WHOLE BLOOD CALCIUM CONTENTS IN FISCHER AND A x C RATS AT CORRESPONDING PERIODS OF THE RESPECTIVE LIFE SPANS

Age as the Percentage of Total Life Span[a]	n	Fischer Ca (in mEq/l)[b]	n	A x C Ca (in mEq/l)[b]	Fischer Ca -A x C Ca[c]	Standard Error of the Difference	p value[d]
< 5	12	3.25 ±.08	6	2.75 ±.35	0.50	0.26	n.s.
50-55	12	3.34 ±.10	5	3.24 ±.10	0.10	0.17	n.s.
>90	4	3.35 ±.03	3	3.20 ±.23	0.15	0.43	n.s.

[a]Maximum total life span for Fischer strain is approximately 23 months and that of the A x C is approximately 30 months.

[b]Calcium values expressed are mean values ± standard errors.

[c]Differences in the mean calcium contents between the two strains are expressed as negative values when the A x C is the higher of the two mean values.

[d]If the p value obtained is <0.05 or <0.01, it is so designated. If the p value is not significant at the 0.05 level it is designated as n.s. The p value was obtained by a weighted 't' test as described in Chapter II (page 24).

TABLE 27

SUMMARY OF COMPARISONS BETWEEN THE TISSUE CALCIUM CONTENTS OF THE TWO RAT STRAINS AT EQUIVALENT PHYSIOLOGICAL AGES

Type of Change	Tissues	Age Expressed as Per Cent of Life Span When Strain Difference Occurs[a]	Strain Exhibiting Significantly Higher Calcium Content[a]
Accumulation Late in Life			
	Kidney	>90	A x C
	Lung	--	--
	Cerebral cortex	< 5	Fischer
Loss Early in Life			
	Skin	< 5	A x C
	Aorta	--	--
	Cardiac muscle	--	--
	Skeletal muscle	--	--
No Apparent Age-related Change			
	Liver	--	--
	Intestinal smooth muscle	50-55 >90	A x C A x C
	Plasma	< 5	Fischer
	Whole blood	--	--

[a](--) = no significant strain difference at any age group compared.

strain differences in calcium content occur are in tissues which show age-related calcium alterations, viz., kidney, cerebral cortex and skin.

Comparison of Overall Mean Calcium Contents

The total mean tissue calcium contents over the entire life span of each rat strain are presented in Table 28. The tissues are listed according to increasing total calcium concentration in the Fischer strain. Table 28 shows that with the exceptions of plasma, liver and kidney, the tissues presented in this study show statistically significant strain differences in overall mean calcium contents. These differences are discussed below in relation to their implications regarding the age-related finding considered earlier.

The strain difference in whole blood calcium concentrations is consistent with both the slight variability observed in each strain and the ability of statistical analyses of such data to identify even small differences. The higher level of whole blood calcium in the Fischer strain will be considered in the Discussion with regard to the maintenance of calcium stores during aging. Plasma calcium was also higher in the Fischer animals, although not significantly so.

The other instances in which strain differences in overall calcium content occur, e.g., cerebral cortex and lung which tend to accumulate calcium during aging, exhibit a higher overall calcium level in the Fischer strain. The Fischer kidney calcium is also higher, but insignificantly so. This suggests that calcium accumulation in certain tissues may be more marked in Fischer than in A x C rats. On the other hand, tissues with an age-related calcium loss show a higher overall calcium level in A x C rats. These findings suggest that the A x C rats may

TABLE 28

COMPARISONS OF OVERALL MEAN CALCIUM CONTENTS OF TISSUES OF FISCHER AND A x C RATS

Tissue	Fischer Strain n	Fischer Strain Mean Ca[a]	A x C Strain n	A x C Strain Mean Ca[a]	Strain with Higher Calcium Content	p value[b]
Whole blood	71	3.24	31	3.04	Fischer	<0.05
Plasma	69	5.13	30	4.88	Fischer	n.s.
Liver	70	6.65	31	6.60	Fischer	n.s.
Cardiac muscle	70	10.22	30	11.20	A x C	<0.05
Skeletal muscle	72	12.75	32	13.74	A x C	<0.01
Skin	72	14.07	32	15.88	A x C	<0.001
Kidney	70	14.91	32	14.25	Fischer	n.s.
Cerebral Cortex	66	15.20	29	13.08	Fischer	<0.05
Intestinal smooth muscle	72	23.02	32	26.06	A x C	<0.001
Lung	69	25.17	32	23.51	Fischer	<0.05
Aorta	67	83.03	28	94.73	A x C	<0.05

[a] Calcium values are in mEq/l for blood fractions; mEq/kg dry weight for other tissues.

[b] p values were obtained from a weighted Student's 't' test as described in Chapter II (page 24).

be capable of maintaining a more desirable calcium balance throughout life than the Fischer animals.

Summary of Results

The major findings which have been described in this chapter are summarized below.

(1) Tissues which tend to accumulate calcium during aging, i.e., kidney, lung and cerebral cortex, do so late in life.

(2) Tissues showing a calcium decline with aging, i.e., skin aorta, cardiac and skeletal muscles, demonstrate the major change fairly early in life.

(3) The major changes in the calcium content of corresponding tissues in the two rat strains tend to occur at different chronological ages. Calcium changes are usually achieved at an earlier age in Fischer than in the relatively longer-lived A x C rats.

(4) When the calcium contents of corresponding tissues in the two rat strains are compared at equivalent physiological ages, less than 20 per cent of the comparisons are statistically significantly different. Fifty per cent of these differences occur in the youngest age groups studied when growth is proceeding rapidly.

(5) Overall mean calcium contents throughout the life-span generally differ in the same tissues of the two rat strains. Animals of the longer-lived A x C strain have the lower overall calcium content in tissues which tend to accumulate calcium during aging. Yet they have a higher calcium content in tissues showing an age-related decline in calcium levels.

CHAPTER IV

DISCUSSION

The present study suggests that calcium levels in different soft tissues of the laboratory rat show different patterns of response to the processes of aging, i.e., some tissues tend to accumulate calcium during aging, other tissues lose calcium, and still others maintain an unaltered calcium level throughout life.

Some of the changes in tissue calcium content which take place with the passage of time appear to be related to the length of the life span of the animal, and rats belonging to strains which have differing life spans exhibit dissimilar age-related calcium changes in the same soft tissues.

The discussion in this chapter considers the four following topics: (1) What are the changes in the dynamic equilibrium of calcium which accompany aging processes? (2) How are the changes which occur in the dynamic equilibrium of calcium related to the changes in the tissue calcium levels observed in this study? (3) What is the relationship of the changes in tissue calcium content to the chronological ages of the rats, and how does this relationship compare with that seen between calcium contents of the tissues and the physiological age range of the animals belonging to the two strains? (4) What are the possible effects of the observed changes in calcium concentration upon the functional

capacity of the tissues involved?

Aging Changes in the Dynamic Equilibrium of Calcium

The dynamic equilibrium of calcium in the mammalian body is a function of the homeostatic regulation of the blood calcium content, the balance between calcium absorption and excretion, and the deposition or removal of calcium in relation to the large pool of this cation in skeletal tissue. The integration of these various mechanisms bears directly upon the calcium levels found in the soft tissues of the body, and changes in the basic components of the dynamic equilibrium during aging appear to be reflected by the age-related changes in calcium levels which were observed in this study.

Blood Calcium

This investigation has shown that the calcium concentrations of plasma and whole blood are maintained at a constant level throughout life in both strains investigated. This indicates that the monitoring system responsible for regulation of calcium levels in this component of the dynamic equilibrium, e.g., parathyroid glands, remain intact throughout life in these rats.

The maintenance of an effective monitoring system throughout life suggests that the soft tissue calcium changes observed in this study are not a direct result of changes in calcium levels in the blood fractions; that is, any change in the calcium concentration of plasma or whole blood is readily remedied by the homeostatic mechanism regardless of the age of the animal. Therefore, if the blood calcium content increases, the increase is only a transient one and the excess calcium

will be excreted or deposited at some site in the body. The blood system serves the body only as a carrier in respect to calcium removal or deposition, but the blood itself is neither the initial source nor the final reservoir of calcium in the body.

Absorption and Excretion of Calcium

In old rats, both urinary and fecal calcium contents are higher than those observed in young animals (Lengemann, 1959; Henry and Kon, 1953; Marcus and Wasserman, 1966). These investigators have shown that the increased calcium which is excreted has as its source the calcium lost from the skeleton during aging, and have verified the fact that the increased excretory calcium is mainly of endogenous origin.

Although it is believed by some investigators that the increased fecal calcium in old rats is due to a decline in the ability of the gut to absorb calcium efficiently (Henry and Kon, 1947; Lemaire, 1968), definitive studies have shown that age has little effect on calcium absorption by the gut when measured by both radioisotopic and precipitation methods (Draper, 1964; Haavaldsen and Nicolaysen, 1965).

Rats receiving a diet containing an adequate amount of calcium take up this cation by an active process in the duodenal region of the small intestine only, but if the diet is low in calcium, other segments of the gut become capable of actively transporting calcium both *in vitro* (Schachter *et al.*, 1966) and *in vivo* (Krawitt and Schedl, 1968). The active transport mechanism by the small intestine is apparently maintained throughout life, because the unabsorbed percentage of calcium from the diet is the same in young and old animals (Draper, 1964).

This ability of the gut to absorb calcium by an inherent active

process is supported by the findings of Kane, Lovelace and McCay (1949) and Haavaldsen and Nicolaysen (1965) who have shown that neither high nor low levels of Vitamin D affect calcium absorption in rats which are over four to six months old.

Evidence that the duodenum is the site of the active transport of calcium has appeared in papers by Nunn and Ellert (1967) and Nordin (1968). These workers have shown that the calcium content of the mucosa in the duodenal region of rats is lower than that in the mucosa of the more distal segments of the small intestine. This suggests that the duodenal region removes calcium by an active process which differs in certain aspects from that operating in other parts of the gut.

The efficiency with which calcium is removed from the gut appears to be unrelated to the age of the animal, and the homeostatic mechanism for the regulation of blood calcium levels is unaffected by aging. Consequently, the changes in tissue calcium content observed in this study must involve the other major contributor to the dynamic equilibrium of calcium, viz., the skeletal system.

Bone Calcium

Hansard, Comar and Plumlee (1951) have shown that up to the age of about 6 months, a large amount of calcium--much of which originates from the extracellular fluid of soft tissues--is deposited in the rat skeleton at a fairly constant rate. By the age of 16 months, however, both the amount and rate of this calcium deposition is significantly decreased. The pattern of skeletal calcium deposition observed by Hansard's group was also recently shown by McBroom, Cornelison and Weiss (1966).

Draper (1964) has shown that after adulthood is reached in rats, the catabolism of skeletal tissue is significantly increased and the animal loses progressively more calcium from its bones as aging processes continue. This process of greater skeletal catabolism, coupled with the decreased calcium uptake by the bony tissue, tends to increase the calcium concentration in blood, a situation which must be rapidly remedied by the homeostatic monitoring system. The excess calcium removed from the blood is, in part, excreted, but some of it is apparently deposited in soft tissues.

Correlation of Dynamic Equilibrium with Changes in Tissue Calcium Contents

Calcium Accumulation

The most important changes in tissue calcium levels during aging are probably those in which the calcium concentration increases as the animal becomes older. Weller (1956) has shown *in vitro* that certain tissues take up calcium to an extent which is dependent upon the amount of calcium presented to them. Indeed, increased catabolism of the skeleton which accompanies old age results in more calcium being circulated through the body. Although much of the excess calcium is excreted, portions of it appear to be deposited in non-osseous tissue.

Carvalho, Sanui and Pace (1965) reported that cellular and nuclear membrane structures which are composed essentially of lipoprotein material can bind considerable quantitites of divalent cations, and Lengemann (1959) suggested that there are specific sites on the membranes that appear to bind calcium exclusively. Binding of large amounts of calcium at membrane sites can impair the functional capacity of the cell (Wallach *et al.*, 1966;

Selye, 1962), a concept which will be discussed later.

This study has shown that the tissues which accumulate calcium during aging, i.e., kidney, lung and cerebral cortex, do so late in life at a time when skeletal catabolism is reported to be increased. The calcium which is deposited in the soft tissues is probably derived from the skeletal tissues of the animal, via the blood circulating through these soft tissues.

Although all the tissues of the body receive blood which is carrying calcium derived from the catabolism of bone, it is interesting to note that those tissues in which calcium seems to accumulate in old age receive a relatively large percentage of the cardiac output. Therefore, in absolute terms, these tissues are subjected to more excess calcium than most other tissues of the body. The finding that the plasma and whole blood calcium concentration is higher in the Fischer rats--when considered as an overall level throughout life--could be indicative of a higher rate of skeletal resorption in that strain.

Factors other than increased skeletal catabolism seem to contribute to the calcification of certain tissues, one of which is probably the phenomenon of parenchymal cell loss from the functional tissue mass in old age.

In kidney, the loss of functional cells has been reported to accompany aging (Lowry et al., 1946). According to the theory proposed by Selye (1962), the loss or damage of cells creates a situation which promotes calcification. In brain tissue, a loss of functional neurons is partially compensated for by replacement of the neurons with non-parenchymal tissue, e.g., glial cells (Hahn, 1966). This cell replacement, however,

compensates for neuronal loss only from the standpoint of cell numbers, and not from the standpoint of functional capacity of the aging brain. Streicher (1958, 1959) reports that most of the calcium increase in the brain tissue of aging rats occurs in the non-parenchymal tissue in the form of calcium phosphate. Consequently, an increase in the amount of non-parenchymal tissue during aging can contribute to the increased calcium content observed in the cerebral cortex in the present study.

Calcium Loss

According to evidence presented earlier, those tissues which exhibit a decreased calcium content during the life span do so during the stages of growth and development, rather than at a period of life which corresponds to senescence. As pointed out previously, the rat skeleton is capable of taking up a sizable amount of calcium derived from the extra-cellular fluid of non-osseous tissue during growth. During the period of growth from infancy to adulthood, most tissues exhibit a decrease in the percentage of total water content, the loss occurring mainly from the extracellular fluid compartment (Andrew et al., 1959; Irvine, Farrelly and Fraser, 1964; Vernadakis and Woodbury, 1964).

Because the extracellular fluid is in Donnan equilibrium with plasma, the loss of extracellular volume from the tissues results in the loss of ionic constituents of the extracellular fluid. The calcium lost through this mechanism during periods of growth is probably taken up by the skeletal tissue, owing to the high affinity of the skeleton for calcium during that period of life.

Processes other than this shift of calcium from extracellular tissue fluid to bone probably participate in the observed decline in

calcium content of certain tissues. An example of such a possibility has been shown for skin. Boros-Farkas, Spichtin and Verzar (1967) showed that soluble collagen has more affinity for calcium than does insoluble collagen, and in rat skin, the percentage of soluble collagen decreases by over 80 per cent from the age of one month to two years. This decrease in the soluble collagen content of the skin, coupled with the water loss which occurs early in the life of the rat, can probably explain the loss of calcium from rat skin during aging, since both a decrease in extracellular fluid volume and a decreased affinity for calcium accompany growth and aging.

In the cases in which tissues lose calcium during aging, the contention of Lowry et al., (1942) seems to hold. These authors suggested that chemical changes during aging are the result of changes in the proportions of various tissue compartments, rather than actual changes in the chemical composition of a given compartment. On the other hand, this explanation does not apply as readily to the calcium accumulation observed in certain tissues, because the accumulation does not occur when major tissue compartmental changes are thought to take place.

Relationship of Tissue Calcium Changes to Chronological and Physiological Age

Chronological Age

Among those tissues which tend to accumulate calcium during aging, the lungs and cerebral cortex of the A x C animals show slower rates of calcium buildup than the corresponding tissues in the Fischer rats. In

both of these tissues, the Fischer animals exhibit a very rapid and quantitatively large increase in calcium content fairly late in life, with the calcium content at that time being significantly higher than at any previous age. This finding suggests an important difference. In Fischer rats there appears to be a major breakdown of the calcium equilibrium, probably at the skeletal level, resulting in a large amount of calcium release and its subsequent deposition in those tissues which show an accumulation of calcium. In the longer-lived A x C strain, the same process appears to occur at a slower rate and to have its onset at a later chronological age.

In each tissue which exhibits an age-related change in calcium content, the change occurs at ages which are probably not chronologically equivalent in the two rat strains. Moreover, most of these strain differences disappear if the calcium levels in the two strains are expressed in terms of physiological age, i.e., as a percentage of the respective maximum life spans.

Physiological Age

Few strain differences are evident when calcium contents of corresponding tissues are expressed as a function of the physiological age, and many of the differences occur only in comparisons made between young animals. Thus, it appears that as the animals of the two strains progress through their respective life spans, they become more comparable to each other from the standpoint of calcium metabolism at the tissue level. A major difference may lie in the rate at which rats of the two strains approach a common level of tissue calcium content. For this reason, the comparison of age-related changes in animals with different

life spans is more meaningful if the ages are expressed in a manner which takes the total life span into account.

Chronological age, however, cannot be ignored in such a study of aging phenomena in animals having differing longevities, since subtle differences such as rates of change of various parameters can be easily overlooked if all comparisons are based on physiological age alone.

The incorporation of both chronological and physiological age relationships into this study has led to two major conclusions about the comparative aspects of the calcium changes in the two rat strains. Firstly, when considered from the standpoint of the total life spans, the two rat strains show little divergence with regard to the tissue calcium status throughout life. Secondly, when the calcium status of the tissues of the two strains is viewed in terms of specific age levels, the longer-lived rats appear to maintain a mature level of tissue calcium content for a longer time than do animals of the shorter-lived strain.

Application of the Tissue Calcium Changes to Various Functions

The calcium buildup which seems to occur in the lungs and cerebral cortex during aging may have detrimental effects on the functional capacity of these vital organs. The fact that this buildup occurs very rapidly in the Fischer strain may contribute to the relatively short life span of these rats.

Weller (1956) states that a change in calcium retention by a tissue probably involves the bound form predominantly, a fact that suggests changes in various cellular constituents which combine with

calcium ions. These cellular constituents probably include membrane structures whose functions may be impaired by large amounts of calcium deposition. For example, the cell membranes of the cerebral cortical neurons must maintain the capacity for rapid ion exchange in order to maintain chemical gradients necessary for electrical activity, a capacity which is adversely affected by high levels of calcium (Wallach et al., 1966). In addition, the functions performed by the lungs and kidneys depend to a large extent upon the passage of materials across their cell membranes and, therefore, may also be impaired by high calcium levels.

If calcium levels become sufficiently elevated, the physical properties of the tissue, e.g., the elasticity of the lung, may even be affected to an extent which impairs the functional capacity of the tissue.

Finally, according to Selye (1962), high calcium levels can augment the formation and hardening of intercellular cementing substance to the extent that cells in the immediate area are functionally isolated from adjacent cells, the result being a loss of the capacity for intercellular communication. If this, or any of the conditions described above, become extensive enough, the function of entire organs and the organism as a whole may be imperiled. It must also be recognized that the accumulation of calcium by certain tissues may be the result of other age-related phenomena which simply predispose the tissues to subsequent calcium buildup.

The loss of calcium from various tissues probably has much less effect on the functional status of the tissue than does calcium accumulation. The fact that calcium losses generally occur fairly early in life

and before age-related functional impairment is normally observed suggests that these calcium losses do not markedly affect the functional status of the organism.

Since calcium losses incurred by various tissues early in life are thought to be at the expense of the extracellular compartment, the loss of total calcium is probably relatively small. For example, in both cardiac (Scott, 1932) and skeletal muscle (Cotlove et al., 1951; Scott and Packer, 1939), only a small percentage of the total calcium is located in the extracellular portion of the tissues.

Andrew et al. (1959) believe that skeletal muscle function declines during aging due to a loss of functional cells. This condition does not appear to be related to the pattern of calcium loss in the present study because Hines and Knowlton (1937) and Neskovic et al. (1965) have shown that the cell loss seen during experimental muscle atrophy is accompanied by an increased calcium concentration in the atrophied tissue.

In summary, it would appear that the changes in soft tissue calcium contents which have the most marked effects on the functional capacity of a tissue, organ, or organism, are those in which the calcium content increases with advancing age. Thus in different strains of the same species which have different life spans, the shorter-lived animals exhibit a marked calcium accumulation in certain vital organs, e.g., brain tissue, at a more rapid rate and at an earlier chronological age than does the correspondingly longer-lived strain. However, based on a small number of extremely old animals, the calcium accumulation in kidney appears to depend upon the length of life rather than to exert any effect upon the life span.

CHAPTER V

SUMMARY AND CONCLUSIONS

In an attempt to elucidate the relationship of age to tissue calcium levels, the calcium concentrations were determined in eleven soft tissues extracted from 104 highly inbred rats at various ages throughout life. The subjects for this study were rats belonging to two highly inbred strains which exhibit differing average life spans. Comparisons were made between the two strains in an attempt to correlate the length of life to the tissue calcium levels during aging.

The tissues in which calcium concentrations were measured were prepared by dry ashing, and the calcium analyses were performed by atomic absorption spectrophotometry.

This study produced the following findings and conclusions:

(1) Certain tissues tend to accumulate calcium during aging, viz., kidney, cerebral cortex, and possibly lung, and this accumulation occurs fairly late in the life of the animal.

(a) The kidney exhibits a pattern of progressive calcification which suggests that the process is dependent on the length of life of the animal, i.e., the longer-lived animals show a significantly greater amount of calcium accumulation than the shorter-lived animals.

(b) The lung and cerebral cortex show patterns of altered calcium content which are marked by a rather sudden onset and

a rapid increase in the shorter-lived rats, whereas these tissues show a more gradual calcium buildup in the longer-lived strain. This finding implies that the rapid calcification of these vital tissues could conceivably contribute to the relative brevity of the life span in rats of the shorter-lived strain.

(2) Certain tissues, i.e., skin, aorta, cardiac and skeletal muscles, exhibit a decline in calcium content fairly early in life. It is suggested that the loss of calcium from soft tissues is probably a process more closely related to growth and development than to senescence.

(3) Liver, intestinal smooth muscle, plasma and whole blood show no changes in calcium concentration throughout life in either rat strain. It is suggested that the monitoring system for blood calcium level, as well as the level of calcium handling by the gut, is probably maintained well throughout the life span of the animals. In addition, other investigators who have studied liver calcium content as a function of age attach little, if any, functional significance to the response of liver calcium content to aging processes (Lowry and Hastings, 1942; Griswold and Pace, 1957).

(4) In the tissues studied, the major changes in calcium content seem to occur at somewhat different chronological ages in the two strains, i.e., the two strains exhibit different patterns of tissue calcium alteration when viewed from the standpoint of chronological age.

(5) Comparisons of calcium levels in corresponding tissues at equivalent physiological age ranges in the two strains, i.e., at ages which represent equivalent percentages of the respective maximum life spans, show that relatively few strain differences occur in the calcium levels. The

majority of the differences take place at the youngest age levels studied.

The findings described above suggest that the changes in soft tissue calcium concentrations in the two rat strains differ in some instances with regard to the rate of the change which occurs, but not with regard to the direction of the change. Most of the changes are not comparable in a temporal sense, but when expressed in terms of the total life span, the two strains exhibit tissue calcium changes which are usually quite similar in pattern and magnitude.

The idea that the two strains do show certain differences in time-related tissue calcium levels is supported further by the results of comparisons between mean overall calcium contents of corresponding tissues. These comparisons show that the tissues in which a calcium accumulation was observed have a consistently higher overall mean calcium content in the Fischer rats, whereas those tissues from which calcium is lost during aging have a higher overall mean calcium content in A x C rats.

The findings and conclusions of this study suggest that the longer-lived A x C animals control their calcium metabolism more efficiently than do the animals of the shorter-lived Fischer strain. This implies that the rats remove calcium from certain tissues at a fairly early age; this calcium is probably deposited in the skeleton. The longer-lived A x C rats seem to release skeletal calcium more gradually, as evidenced by the slower and more progressive nature of the soft tissue calcium accumulation in these longer-lived rats.

The rapid increase in calcium content in some of the vital soft tissues, e.g., cerebral cortex in the shorter-lived Fischer strain, is a

factor which could contribute to the establishment of a definite life span. This process of rapid calcium accumulation must be detrimental to the functional status of the animal, or at least be concomitant with some loss of function. Since this process does not occur to such an extent in the A x C rats, the relatively long life span of this strain may reflect a more effective regulation of calcium equilibrium.

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APPENDIXES

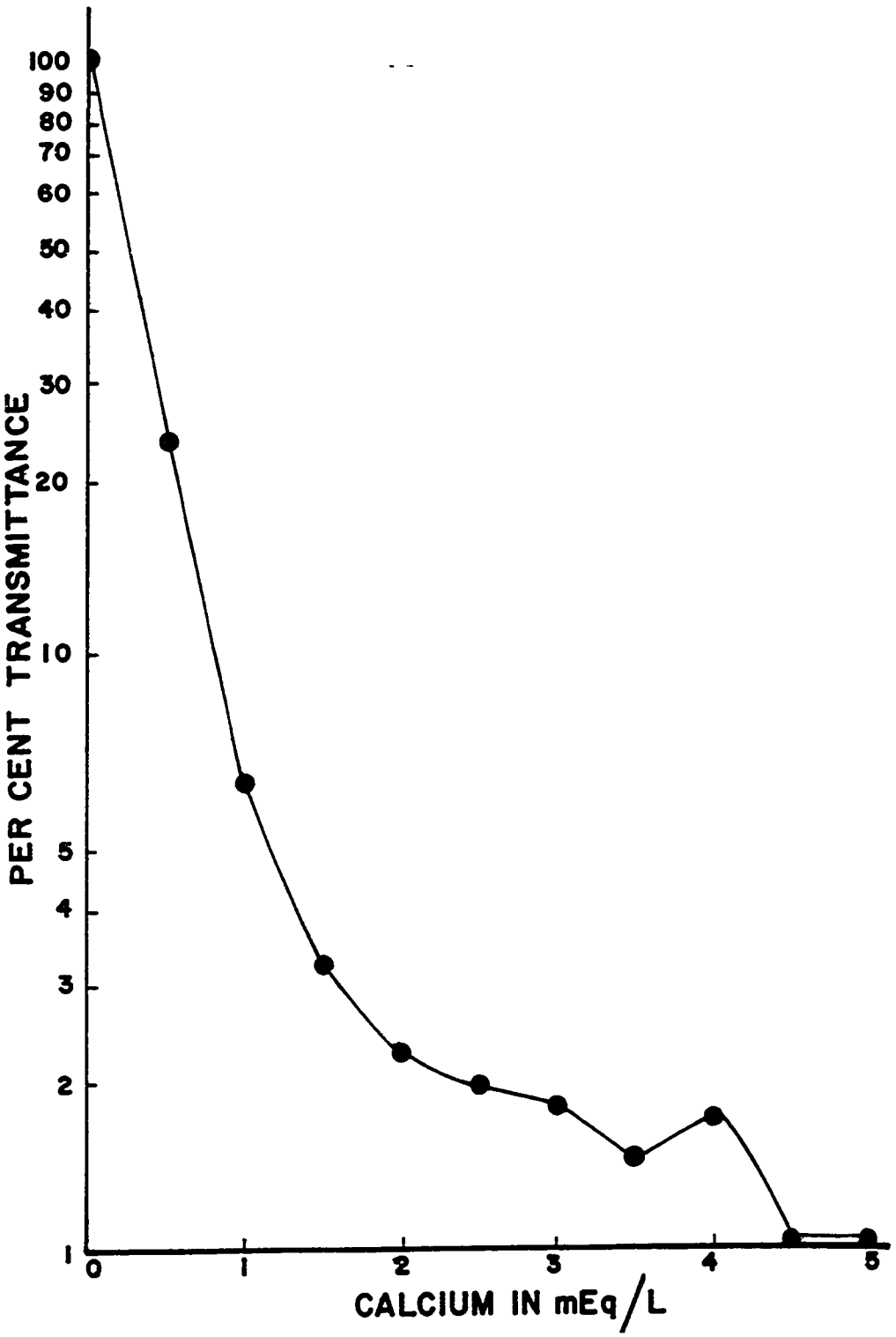
APPENDIX I

COMPOSITION OF DIETS

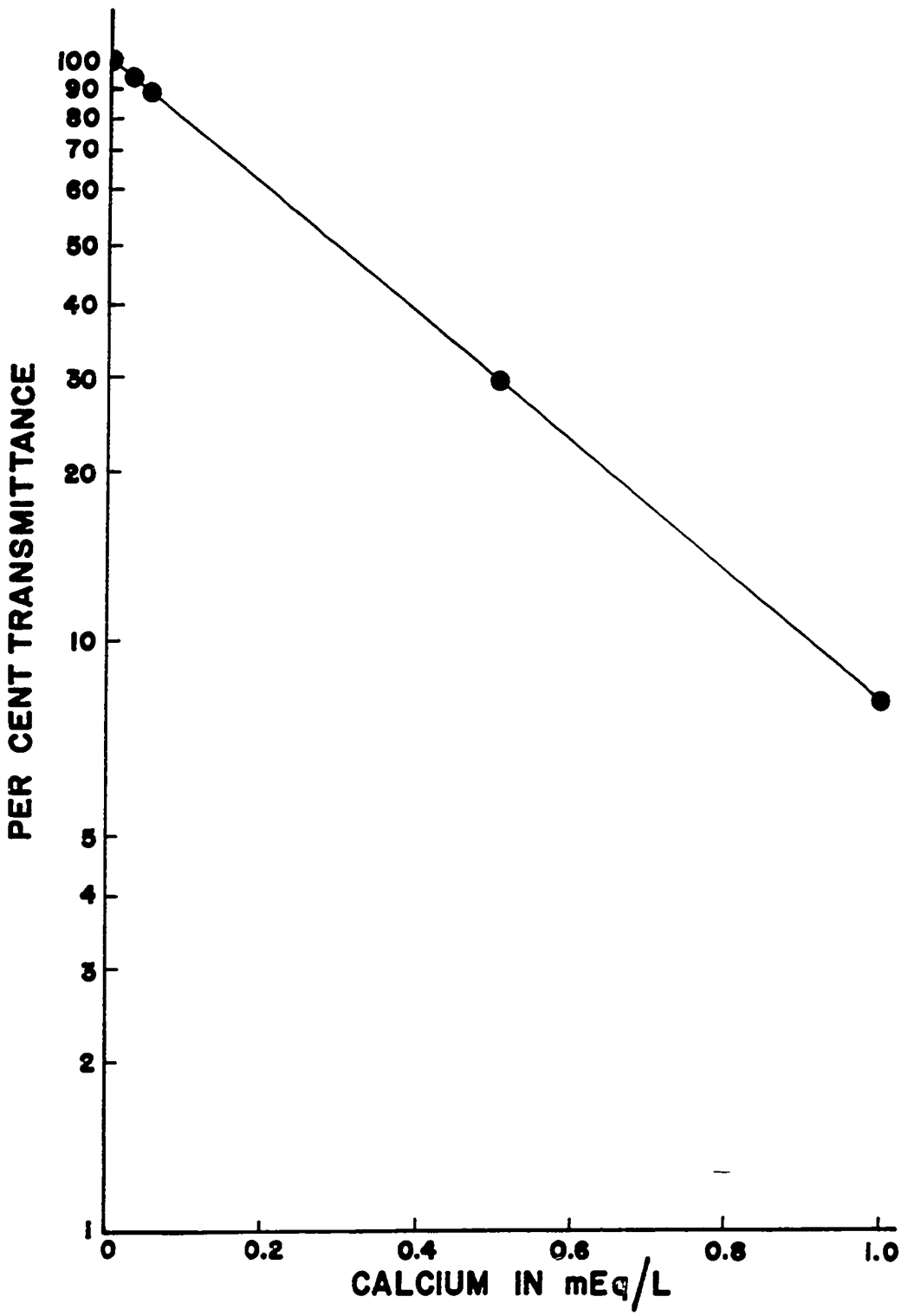
Nutrients	Rockland Diet (Teklad)	Lab Chow (Purina)
Fiber (%)	3.34	5.20
Ash (%)	8.64	7.30
Carbohydrate (%)	55.67	55-60
Fat (%)	6.53	4.53
Protein (%)	24.69	23.40
Lysine	1.52	1.41
Cystine	0.37	0.32
Methionine	0.477	0.43
Tryptophane	0.27	0.28
Arginine	1.47	1.38
Histidine	0.62	0.62
Isoleucine	1.30	1.22
Leucine	2.04	1.80
Phenylalanine	1.07	1.03
Threonine	0.95	0.94
Valine	1.29	1.24
Glycine	1.13	1.26
Minerals (%)		
Chlorine	0.72	0.51
Sodium	0.58	0.49
Potassium	0.86	0.82
Calcium	1.44	1.30
Phosphorus	1.17	0.94
Magnesium	0.26	0.26
Manganese	0.0152	0.0051
Iron	0.0319	0.0198
Zinc	0.0063	0.0058
Copper	0.00135	0.0018
Cobalt	0.000137	0.00004
Iodine	0.00025	0.00017
Fluorine	Trace	0.0035
Vitamins (per 100 g)		
Vitamin A	1385 U.S.P. Units	1200 I.U.
Thiamin B-1	1475 mcg	1770 mcg
Riboflavin B-2	721 mcg	850 mcg
Pyridoxine B-6	997 mcg	380 mcg
Pantothenic acid	2264 mcg	2480 mcg
Vitamin D	507 U.S.P. Units	530 I.U.
Vitamin E	3.84 I.U.	6.57 I.U.
Niacin	10.89 mg	11.03 mg
Carotene	831 mcg	650 mcg
Choline	159 mg	240 mcg
Folic acid	398 mcg	590 mcg
Vitamin B-12	2.84 mcg	2.25 mcg
Menadione	110 mcg	-
Biotin	-	7.00 mcg

APPENDIX II

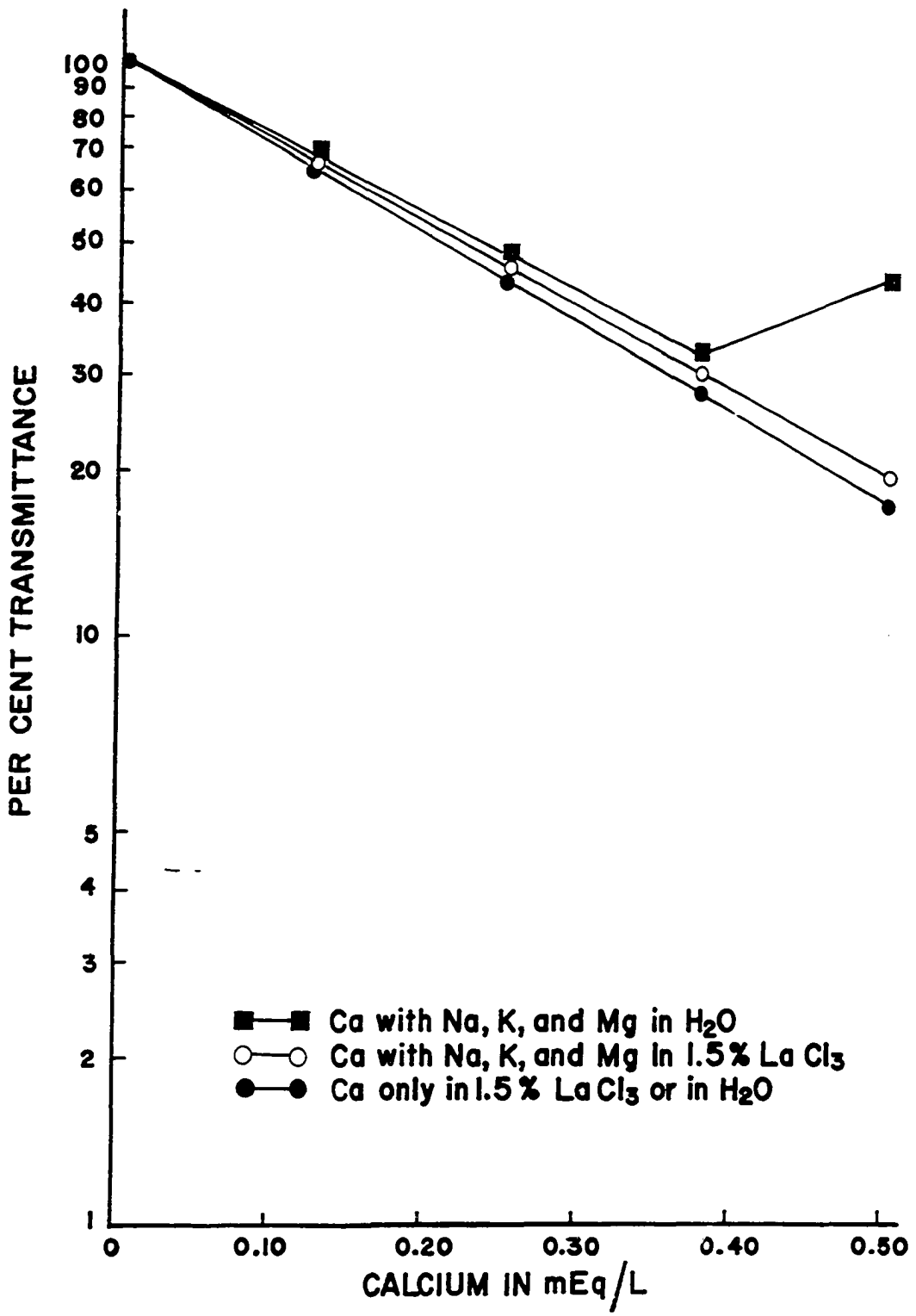
PERCENT TRANSMITTANCE READINGS OF VARIOUS CALCIUM CONCENTRATIONS TO DETERMINE LINEAR RANGE



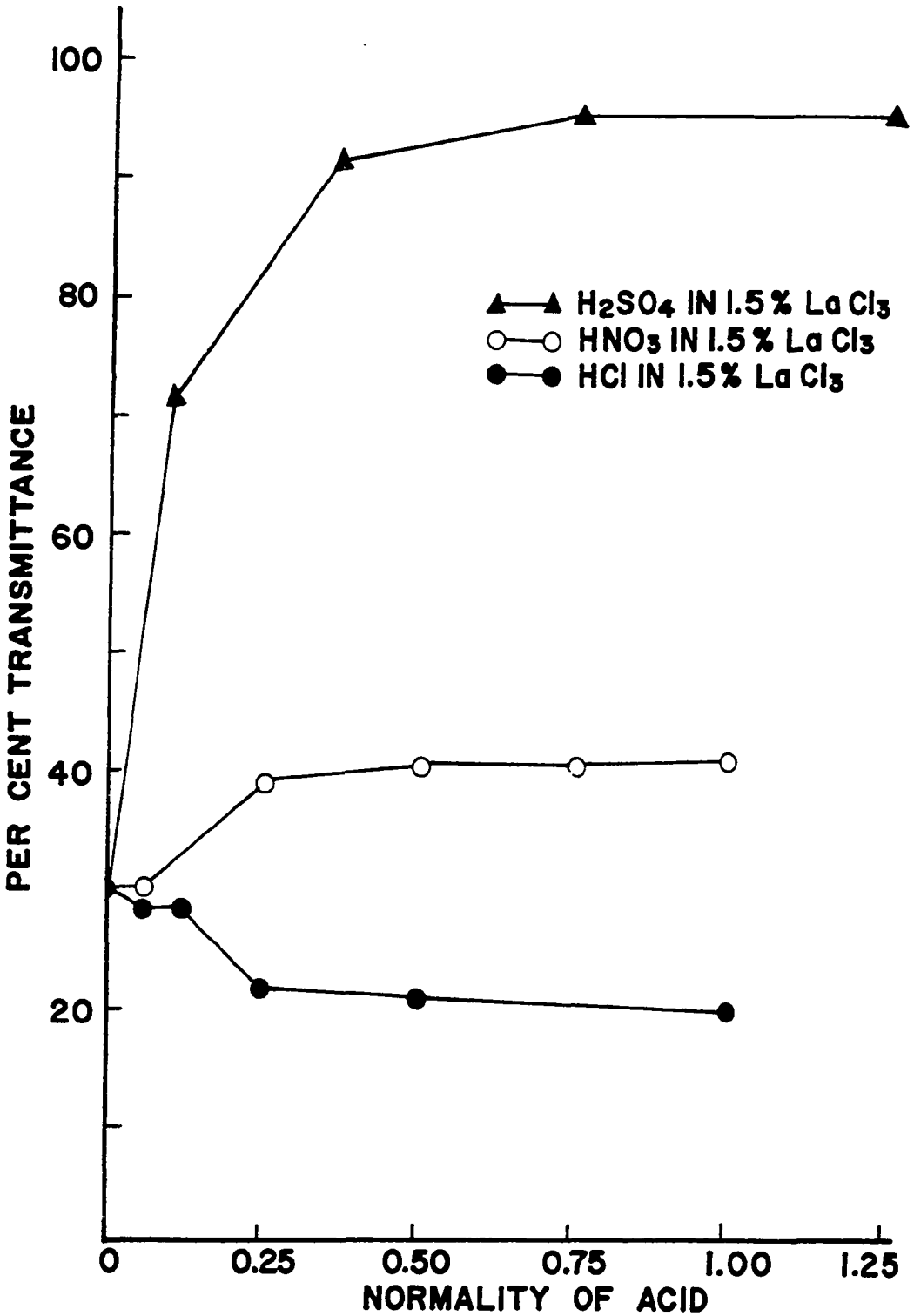
PERCENT TRANSMITTANCE READINGS OF VARIOUS CALCIUM CONCENTRATIONS SHOWING DETECTABILITY LIMITS



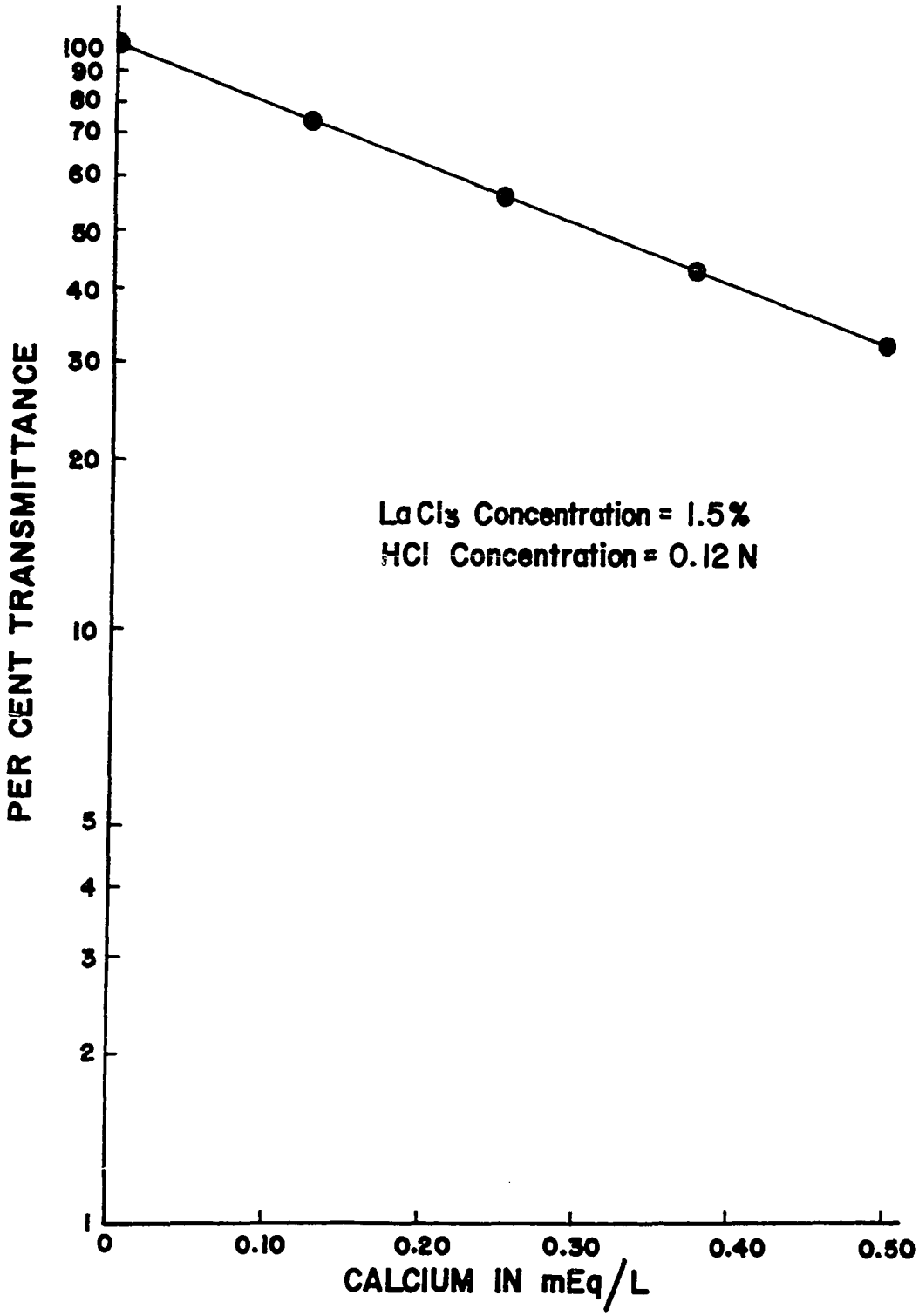
EFFECTS OF INTERFERING ELEMENTS ON CALCIUM DETERMINATION IN THE PRESENCE AND ABSENCE OF LANTHANUM CHLORIDE



EFFECTS OF SAMPLE NORMALITY ON PERCENT TRANSMITTANCE READINGS OBTAINED FROM A STANDARD CALCIUM SOLUTION OF 0.50 mEq/L



TYPICAL STANDARD PLOT FOR DETERMINATION
OF CALCIUM CONCENTRATION



APPENDIX III

SAMPLE CALCULATIONS OF CALCIUM CONTENT

Blood Fractions

Assuming that a reading of 49 per cent transmittance is obtained from a sample of whole blood which has been diluted by a factor of ten:

49% T = 0.312 mEq/L (from standard curve in APPENDIX II) and,

0.312 mEq/L x 10 (dilution factor) = 3.12 mEq/L.

Soft Tissues

Assuming that a reading of 85 per cent transmittance is obtained from a sample of liver tissue having a dry weight of 290.0 milligrams and a final sample volume of 25.0 milliliters:

85% T = 0.073 mEq/L (from standard curve in APPENDIX II),

$$\frac{0.073 \text{ mEq}}{1000 \text{ ml}} = \frac{n \text{ mEq}}{25.0 \text{ ml}}, \quad n = 0.001825 \text{ mEq}/25.0 \text{ ml}, \text{ and}$$

$$\frac{0.001825 \text{ mEq}}{290.0 \text{ mg dry wt.}} = \frac{x \text{ mEq}}{1 \times 10^6 \text{ mg dry wt.}},$$

Thus X = 6.29 mEq/kg dry tissue wt.

SAMPLE ANALYSIS USING NEW MULTIPLE RANGE TEST (according to Kramer, 1956)

The New Multiple Range Test is designed to allow comparison of each of a set of ranked mean values with each other mean value, and the procedure is especially well suited for data which do not lend themselves to a regression analysis.

Kidney Calcium in A x C Strain

The mean calcium concentrations ($\bar{x}$) in mEq/kg dry tissue of the various age groups are ranked in order of magnitude along with the respective numbers of replications:

Age in months	2	1	16	27-30
$\bar{x}$	12.96	12.98	15.35	18.49
n	6	6	6	3

An analysis of variance is performed on the raw data and the results are presented in Table A.1.

TABLE A.1

ANALYSIS OF VARIANCE

Source of Variation	df[a]	SS[b]	MS[c]	F value
Treatment (among age groups)	3	80.11	26.70	22.25
Error (within age groups)	17	20.32	1.20	...
Total	20	100.43	...	...

[a]Degrees of freedom.

[b]Sums of squares.

[c]Mean squares.

[d]F value obtained is compared with appropriate tabular F (Steel and Torrie, 1960).

SAMPLE ANALYSIS -Continued

When the F value thus obtained is compared with the tabulated F value (3.20 for 3 treatment and 17 error degrees of freedom), the level of significance is found to be less than 0.01 and further testing is necessary. The New Multiple Range Test is applied to the data as described below:

The weighted standard deviation (s) is calculated by the extraction of the square root of the error mean square in Table A.1,

$$s = \sqrt{\text{Error MS}} = \sqrt{1.20} = 1.10$$

Significant studentized ranges (SSR) are then obtained from appropriate tables (Steel and Torrie, 1960), and significant range factors (R'_p) are calculated by multiplication of each SSR by the previously determined value for s (1.10).

p	2	3	4
SSR (17 error df)	4.10	4.30	4.41
R'_p	4.52	4.73	4.85

Significant differences are tested for, beginning with a comparison between the largest mean calcium value (the 27-30 month age group) and the smallest mean calcium value (the two-month age group). In order for the difference to be statistically significant at the 0.01 level,

$$(\bar{x}_{27-30} - \bar{x}_2) \sqrt{\frac{2(n_{27-30})(n_2)}{n_{27-30} + n_2}} \text{ must exceed } R'_4 = 4.85$$

Therefore, $(18.49-12.96)\sqrt{\frac{2(3)(6)}{3+6}} = 5.53 \times 2 = 11.06$, and exceeds R'_4.

SAMPLE ANALYSIS -Continued

Since the value obtained exceeds the significant range factor (R'_4) with which it must be compared, the difference between the two mean values is statistically significant at the 0.01 level and the testing procedure is continued. The next comparison is between $\bar{x}_{27-30}$ and $\bar{x}_1$ with the following value being obtained:

$(18.49-12.98)\sqrt{4} = 5.51 \times 2 = 11.02$, which exceeds the value of R'_3 or 4.73 with which it must be compared, and this difference is, therefore, statistically significant at the 0.01 level. The need for further testing is indicated and the next comparison is between $\bar{x}_{27-30}$ and $\bar{x}_{16}$:

$(18.49-15.35)\sqrt{4} = 3.14 \times 2 = 6.28$, which exceeds the value of R'_2 and statistical significance is again indicated.

The testing is continued until no further significant differences between various mean values are indicated. The continuation of testing in this example would be performed by comparing the second largest mean value ($\bar{x}_{16}$) with all smaller mean values, and the testing pattern continued until a comparison yields no statistically significant value. In this example, statistical significance is indicated until the comparisons reach the level at which $\bar{x}_1$ is compared to $\bar{x}_2$ as shown below:

$(12.98-12.96)\sqrt{6} = 0.02 \times 2.45 = 0.049$, which does not exceed R'_2 or 4.52, the value with which it must be compared, and the analyses of these data are complete.

The findings of analyses such as those described above are presented in Chapter III for each tissue studied, and the form in which these findings are described appears in Table A.2 of this appendix.

SAMPLE ANALYSIS - Continued

TABLE A.2

NEW MULTIPLE RANGE TEST FOR KIDNEY CALCIUM CONTENT IN A x C RATS

Number of Rats (n)	Age in Months	1	2	16	27-30
6	1	12.98[a] ±0.37	-[b]	*[c]	*
6	2	.....	12.96 ±0.33	*	*
6	16	.....	.....	15.35 ±0.64	*
3	27-30	.....	.....	.....	18.49 ±0.32

[a]Mean calcium values for the respective age groups are shown on the diagonal as mEq/kg dry tissue ± standard error of the mean.

[b](-) indicates no statistically significant difference between the two mean values being compared.

[c](*) indicates a statistically significant difference between the two mean values being compared at the 0.05 level.

Table A.2 employs a matrix presentation of the results obtained from the statistical testing procedure. To determine if the tissue calcium content of a given age group differs significantly from that of another age group, locate the age in months in the vertical column so designated and the age in months with which it is to be compared in the row at the top of the table. At the point in the table where the two ages of interest intersect, a symbol appears which denotes either statistical significance or the absence of any significance between the two age groups being compared.